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ORIGINAL ARTICLE

A new role for the P2Y-like GPR17 receptor in the modulation of multipotency of oligodendrocyte precursor cells in vitro

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Abstract Oligodendrocyte precursor cells (OPCs, also called NG2 cells) are scattered throughout brain parenchyma, where they function as a reservoir to replace lost or damaged oligodendrocytes, the myelin-forming cells. The hypothesis that, under some circumstances, OPCs can actually behave as multipotent cells, thus generating astrocytes and neurons as well, has arisen from some in vitro and in vivo evidence, but the molecular pathways controlling this alternative fate of OPCs are not fully understood. Their identification would open new opportunities for neuronal replace strategies, by fostering the intrinsic ability of the brain to regenerate. Here, we show that the anti-epileptic epigenetic modulator valproic acid (VPA) can promote the generation of new neurons from NG2$^+$ OPCs under neurogenic protocols in vitro, through their initial de-differentiation to a stem cell-like phenotype that then evolves to "hybrid" cell population, showing OPC morphology but expressing the neuronal marker βIII-tubulin and the GPR17 receptor, a key determinant in driving OPC transition towards myelinating oligodendrocytes. Under these conditions, the pharmacological blockade of the P2Y-like receptor GPR17 by cangrelor, a drug recently approved for human use, partially mimics the effects mediated by VPA thus accelerating cells' neurogenic conversion. These data show a co-localization between neuronal markers and GPR17 in vitro, and suggest that, besides its involvement in oligodendrogenesis, GPR17 can drive the fate of neural precursor cells by instructing precursors towards the neuronal lineage. Being a membrane receptor, GPR17 represents an ideal "druggable" target to be exploited for innovative regenerative approaches to acute and chronic brain diseases.

Keywords NG2 cells · GPR17 · Neurogenesis · Valproic acid · Oligodendrocyte precursor cells

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Introduction

After embryonic development, quiescent neural stem-like progenitors (NPCs) still persist in two brain's neurogenic niches (i.e., the subventricular zone of the lateral ventricles and the subgranular layer of the hippocampus) and in central nervous system (CNS) parenchyma throughout life (reviewed in [1, 2]). Parenchymal NPCs include subsets of astrocytes that, when activated, re-acquire stem/progenitor cells properties [3], and oligodendrocyte precursor cells (OPCs), also known as polydendrocytes, expressing the membrane chondroitin sulfate proteoglycan NG2 [1, 4]. NG2-positive cells have been originally identified as the progenitors of the myelin-forming cells of the brain and spinal cord (reviewed in [5]) that spontaneously differentiate to mature oligodendrocytes both in vitro and in vivo [6]. The hypothesis that OPCs can behave as multipotent progenitors was raised after the demonstration that NG2 cells purified from early postnatal rat optic nerves have the ability to revert to stem-like cells which eventually

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differentiate to oligodendrocytes, astrocytes, and even neurons [7–9]. Afterwards, with the development of genetic labeling, transgenic mouse models, in particular those carrying the Cre-loxP technology, have become a useful tool for fate-mapping studies to investigate the multipotency of these cells in vivo. For example, following brain ischemia due to permanent middle cerebral artery occlusion (MCAo), generation of both astrocytic and neuronal precursors from NG2+ cells was observed in adult NG2creBAC:ZEG double transgenic mice, in which enhanced green fluorescent protein (EGFP) is expressed in OPCs and their progeny, thus allowing to visualize their final phenotype [10]. However, conflicting data arise from the different transgenic mouse models employed, and the controversy around the multipotency and neurogenic potential of OPCs in vivo is still an open issue [11–15].

Thus, knowledge of the mechanisms underlying the commitment of OPCs towards either myelinating oligodendrocytes or distinct neural lineage is mandatory, but still incomplete. In this respect, we have recently focused our attention on the possible role exerted by the P2Y-like receptor GPR17, which is closely related to both purinergic P2Y and CysLT receptors [16]. Notably, GPR17 is one of the three genes differentially expressed by human adult hippocampal precursor cells when compared to embryonic stem cells [17]. We and others have demonstrated that GPR17 is transiently expressed at specific differentiation stages of OPCs, undergoes a tightly regulated modulation to drive OPC maturation towards fully mature oligodendrocytes, and contributes to their reaction to harmful conditions [18–22].

However, while in the intact CNS GPR17 is almost exclusively expressed in OPCs, this receptor is rapidly induced in cells of the neuronal lineage inside and at the border of ischemic/traumatic lesions in both the MCAo model [16, 23] and in a rodent model of spinal cord injury [24]. Moreover, more recent data have shown that GPR17 regulates the proliferation of a population of doublecortin (DCX)+ neuronal progenitors (neuroblasts) in hippocampal dentate gyrus [25], to suggest that, besides its established role in oligodendrogenesis, GPR17 may also be involved in neuronal specification. On this basis, we raised the hypothesis that GPR17 could regulate the fate of NPCs addressing them to either the oligodendroglial or the neuronal lineages.

This work was specifically undertaken to elucidate the possible role of GPR17 in controlling the switch of OPCs towards a neurogenic fate. To this aim, we purposely set up two culturing protocols known to unveil the multipotency of OPCs in vitro, and we fostered neurogenesis by exposing cultures to the epigenetic anti-epileptic agent valproic acid (VPA), known to promote the generation of new neurons through inhibition of histone deacetylases (HDACs) in progenitor cells [26–28]. In the different experimental conditions, we analyzed the expression of GPR17 and the effect of its pharmacological manipulation on cell progeny. Results show, for the first time, the presence of GPR17 in a "hybrid" cell population that still bore an OPC morphology but already expressed neuronal markers; moreover, blockade of GPR17 by the P2Y antagonist Cangrelor, that also acts as an antagonist at GPR17 [16], partially reproduced VPA-mediated increase in neurogenesis, thus unveiling a new potential pharmacological approach to shift the cell fate of OPCs towards the generation of new neuron-like cells.

Materials and methods

Primary OPC cultures

OPCs were isolated from mixed glial cultures from postnatal day 2 Sprague-Dawley rat cortex, by the shaking method [22] followed by immunopurification [29]. This additional step consists of a negative selection procedure to remove undesired cells (e.g., astrocytes, meningeal cells, and microglia/macrophages) by means of the RAN2 antibody, which does not bind to OPCs [30].

A layer of anti-immunoglobulin antibodies (1 mg/ml anti-IgG, MP Biomedicals, Santa Ana, CA) was adsorbed to empty Petri dishes the day before culture shaking and incubated overnight at 4 °C, thereby improving the subsequent binding of cell type-specific antibody. The following day, mixed cultures were shaken on an orbital shaker for 3–4 h at 200 rpm. In the meantime, IgG-coated dishes were rinsed three times with PBS and incubated for at least 3 h at room temperature (RT) with a solution containing RAN2 antibody (RAN2-Ab, kindly provided by Prof. Carla Taveggia, Axo-Glia Unit, Institute of Experimental Neurology Division of Neuroscience, San Raffaele Scientific Institute, Milan) and consisting in 0.5 ml RAN2-Ab + 6 ml of Minimum Essential Medium with Earle's salts and L-glutamine (MEM, Life Technologies) + 1 mg/ml bovine serum albumin (BSA, Sigma-Aldrich) + 25 mM HEPES pH 7.5 (Sigma-Aldrich).

At the end of shaking, the medium containing the detached cells was collected from each flask in 50 ml sterile conical tubes and centrifuged for 10 min at 290×g. Pellets were resuspended in NM15 Medium containing MEM, 15 % heat-inactivated fetal bovine serum (FBS, Euroclone), 6 mg/ml glucose (Sigma-Aldrich), and penicillin–streptomycin (100 U/ml and 100 μg/ml, respectively; Euroclone) + insulin (5 μg/ml; Sigma-Aldrich), and the cell suspension was incubated at RT in a first RAN2-Ab-precoated plate. After 20 min, floating cells were transferred to a second RAN2-Ab-precoated plate and incubated for additional 20 min at RT. The supernatant was then collected and centrifuged at 290×g for 10 min. The resulting pellet was resuspended in the appropriate medium according to the subsequent protocol (see below). Isolated OPCs were plated onto poly-D,L-ornithine- (final concentration 5 μg/ml; Sigma-Aldrich) coated 13 mm

diameter glass coverslips (15,000 cells/well) for immunocytochemistry or 35 mm diameter Petri dishes (100,000 cells/well) for Western blot analysis.

To verify whether OPCs can generate neurons under a standard protocol of oligodendrocyte differentiation [20–22], cells were plated in Neurobasal medium with 2 % B27 Supplement (both from Life Technologies), 2 mM L-glutamine, 10 ng/ml human platelet-derived growth factor BB (PDGF-BB, Sigma-Aldrich), and 10 ng/ml human basic fibroblast growth factor (bFGF, R&D Systems) to promote proliferation. After 2 days, OPCs were shifted to differentiating medium (i.e., Neurobasal medium lacking growth factors), and either grown under control conditions or exposed to the anticonvulsant agent valproic acid (VPA, 500 μM) for 24–72 h, fixed, and immunostained for GPR17 and the neuronal marker βIII-tubulin (see below).
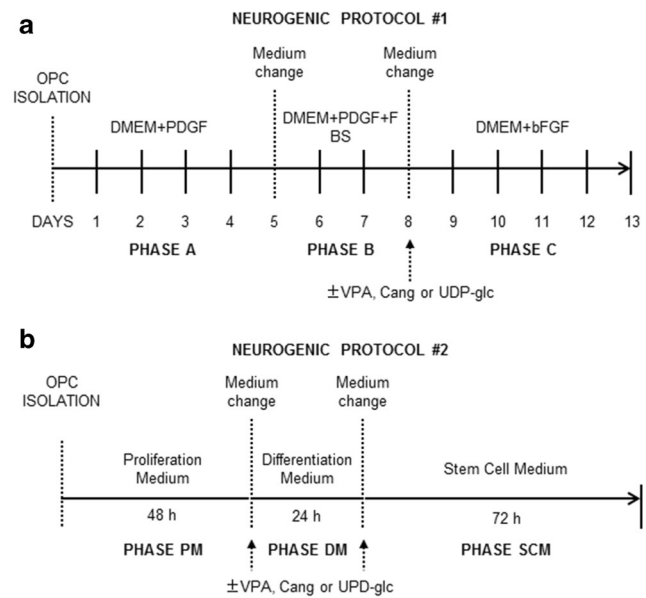
Neurogenic protocols

To test the ability of OPCs to generate neurons, we applied two published protocols claimed to foster OPC transition towards neuroblasts [7, 27].

A three-phase protocol [7] was renamed here as neurogenic protocol #1 (Fig. 1a). Cells were initially maintained for 5 days in DMEM Medium (consisting in DMEM high glucose supplemented with penicillin–streptomycin 100 U/ml and 100 μg/ml, respectively; 1 mM sodium pyruvate; 2.5 μg/ml Fungizone; 2 mM L-glutamine; all purchased from Euroclone) + 10 ng/ml PDGF-BB + B27 supplement (1:50) to induce OPCs proliferation (phase A). Cells were then shifted to DMEM Medium + 10 ng/ml PDGF-BB + B27 supplement (1:50) + 15 % FBS to promote their de-differentiation towards GFAP$^+$ precursors and cultured for 3 days (phase B). Finally, cells were maintained for 5 additional days in DMEM Medium + B27 supplement (1:50) + 10 ng/ml basic fibroblast growth factor (bFGF, R&D) to induce their differentiation along the three neural lineages (phase C).

We then set up an additional neurogenic protocol, renamed here as neurogenic protocol #2 (Fig. 1b; [27]). Cells were exposed for 2 days to Proliferation Medium (i.e., DMEM Medium + 10 ng/ml PDGF-BB + 10 ng/ml bFGF + B27 supplement (1:50); phase PM) and then committed to differentiate into oligodendrocytes by removing mitogens for 1 day (Differentiation Medium; phase DM). Finally, cells were cultured in Stem Cell Medium composed by DMEM/F-12 (Life Technologies), glutamine (1 mM), glucose (25 mM, Sigma-Aldrich), and B27 supplement (1:50) for 3 days (phase SCM), which directly redirect OPCs to the three neural lineages [27].

In both protocols, at the end of the different phases, either cells were fixed with 4 % paraformaldehyde and processed for immunocytochemistry or whole-cell lysates were prepared and analyzed by Western blotting (see "Results" and figures).

Fig. 1 Schematic representation of the two neurogenic protocols utilized in this study (see text for details and drug concentrations). In neurogenic protocol #1 (a; [7]), pharmacological treatments were performed during phase C, whereas in neurogenic protocol #2 (b; [27]) exposure to the selected drugs was started at the beginning of phase DM up to the end of the experimental protocol. *PM*, proliferation medium; *DM*, differentiation medium; *SCM*, stem cell medium; *VPA*, valproic acid; *Cang*, Cangrelor; *UDP-glc*, UDP-glucose

Pharmacological treatments

In either neurogenic protocol, we have verified if and how the exposure to various pharmacological agents (including GPR17 receptor ligands) can modulate OPCs plasticity and their differentiation to neurons. In particular, we have utilized the non-selective GPR17 agonist UDP-glucose (UDP-glc; 100 μM [16, 20–23, 31–34]) and antagonist Cangrelor (Cang; 10 μM), in parallel to VPA (500 μM). All reagents were obtained from Sigma-Aldrich, except for Cangrelor that was a kind gift of The Medicines Company (Parsippany, NJ, USA). In neurogenic protocol #1, cells were treated with the different pharmacological agents during phase C only, whereas in neurogenic protocol #2 cells were treated during both phase DM and SCM (see Fig. 1a, b).

Immunocytochemistry, image processing, and data analysis

Fixed cells were subjected to immunocytochemistry as previously described [20]. The following antibodies and final dilutions were used: primary antibodies: rabbit anti-GFAP (1:600, Dako Italia, Cernusco sul Naviglio, Milan, Italy), mouse anti-βIII-tub (1:1,000; Promega, Milan, Italy), mouse anti-NG2 (1:200; Abcam, Cambridge, UK), and rabbit anti-GPR17 (1:100; Cayman Chemical Company, Ann Arbor, MI); secondary antibodies: goat anti-rabbit or goat anti-

mouse antibodies conjugated to AlexaFluor®488 or AlexaFluor®555 (all 1:600, 1 h at RT; Life Technologies). Nuclei were then labeled with the fluorescent dye Hoechst-33258 (1:10,000 in PBS; Life Technologies) and coverslips were mounted in Dako Fluorescence Mounting Medium (Dako).

Cells were finally analyzed by a fluorescent microscope (Zeiss, Jena, Germany). In each coverslip, the total number of cells, evaluated by counting Hoechst-33258[+] nuclei, and the number of GPR17-, βIII-tubulin, GFAP-, or NG2-positive cells were determined in 20 randomly chosen optical fields under a ×40 magnification. The total number of cells counted for each experimental condition was between 300 and 500.

To evaluate the intensity of GPR17 staining in OPCs cultured in standard differentiating conditions, fluorescent images were captured under a Zeiss Axiovert 200M microscope (Carl Zeiss) equipped with a CCD camera module at ×40 magnification. Densitometric analysis was performed after converting the fluorescent signals to gray-scale values. The mean gray value in 10 randomly chosen optical field/coverslip was measured by the ImageJ software.

Western blotting analysis

Whole-cell lysates were prepared and analyzed by Western blotting as previously described [35]. Cell pellets were homogenized on ice in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5 % sodium deoxycholate, 1 % Triton, 0.1 % SDS) added with 1:100 protease inhibitor cocktail (Sigma-Aldrich). Thirty-microgram aliquots from each protein sample were loaded on 11 % sodium-dodecylsulfate polyacrylamide gels and blotted onto nitrocellulose or PVDF membranes (Bio-Rad Laboratories, Milan, Italy). Membranes were then saturated with 10 % non-fat dry milk in Tris-buffered saline (TBS; 1 mM Tris-HCl, 15 mM NaCl, pH 8) for 1 h at RT and incubated overnight at 4 °C with mouse anti-synaptic vesicle 2 (SV2, 1:2,000) and anti-βIII-tub (1:1,000, Promega, Milan, Italy) or rabbit anti-synaptotagmin (Syt, 1:2,000; anti-SV2 and anti-Syt are a kind gift of Dr. C. Verderio, CNR, Milan), anti-CNPase (1:250, Santa Cruz), and anti-GPR17 primary antibodies (1:100; home-made polyclonal antibody from Dr. Patrizia Rosa, CNR, Milan) all in 5 % non-fat dry milk in TBS. Membranes were then washed in TBS-T (TBS plus 0.1 % Tween20®), incubated for 1 h with goat anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:4,000 or 1:2,000, respectively in 5 % non-fat dry milk in TBS; Sigma-Aldrich). Detection of proteins was performed by enhanced chemiluminescence (ECL, Amersham Biosciences, Milan, Italy) and autoradiography. Non-specific reactions were evaluated in the presence of the secondary antibodies alone.

Luciferase reporter assay

For the analysis of promoter induction, the Oli-neu murine cell line was cultured in Sato medium containing 1 % horse serum. The day after seeding, cells were transfected with a reporter construct containing an active region of the human GPR17 promoter, as previously described [36]. Exposure to 1 mM VPA was started the following day and lasted 48 h. The Dual Luciferase Reporter Assay (Promega) was performed according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using the GraphPad Prism5 software. Differences between experimental conditions were calculated using either unpaired two-tailed Student's t test or one-way ANOVA followed by the Bonferroni's post hoc test. A p value <0.05 was considered as significant.

Results

NG2[+] OPCs are multipotent cells, and their multipotency is unveiled by specific neurogenic protocols

When cultured in differentiating conditions, NG2^{+} OPCs progressively maturate to myelinating oligodendrocytes [20–23]. To verify whether their intrinsic multipotency can be unveiled under these standard culturing conditions, we isolated rat OPCs from mixed cortical glial cell cultures, grew them for 24–72 h under control (CTR) conditions or in the presence of 500 μM VPA (an anti-epileptic agent known to stimulate neurogenesis by inhibiting HDACs; [26–28]), and counted the number of βIII-tubulin (βIII-tub)[+] cells.

Very few βIII-tub^{+} cells were observed at any of the time points tested either in CTR cultures or following exposure to VPA (Fig. 2a), with a trend to decrease with time in culture. When this cell population was evaluated as percentage of the total number of cells (Fig. 2b), values between 0.60 and 0.35 % were obtained (Fig. 2c), thus suggesting that these culturing conditions are not able to switch OPCs from their oligodendrocyte fate to a neurogenic one, not even in the presence of a known pro-neurogenic agent like VPA. Thus, to unmask the latent ability of OPCs to generate neurons, we grew them according to two protocols, renamed here neurogenic protocol #1 [7] and neurogenic protocol #2 ([27]; see "Materials and methods" and Fig. 1). At the end of each phase, we performed immunocytochemical analysis to characterize the composition of the cell population.

Concerning protocol #1, at the end of phase A, the majority of cells were NG2^{+} OPCs, while only few GFAP[+] astrocytes were observed (Fig. 3; see histograms in a and representative picture in b). A significant population of cells expressed
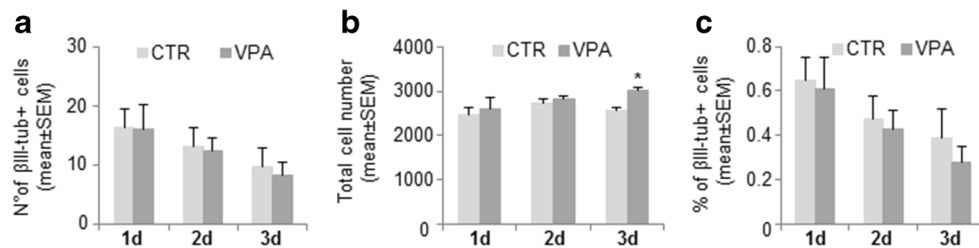
Fig. 2 VPA does not promote the generation of βIII-tubulin⁺ neurons from OPCs grown in standard differentiating conditions. **a–c** Histograms showing the number of βIII-tubulin⁺ cells (**a**), the total number of cells (as evaluated by counting Hoechst33258-stained nuclei; **b**), and the percentage of βIII-tubulin⁺ cells over the total cell population (**c**) in OPC cultures grown under standard differentiating conditions for 1–3 days. Cultures have been grown either under control condition (CTR) or exposed to 500 μM VPA. Results are the mean of 3 coverslips/condition from 1 representative experiment. *$p < 0.05$ compared to CTR, unpaired two-tailed Student's t test

GPR17 (Fig. 3a), with more than 60 % of GPR17⁺ cells also co-expressing NG2, thus confirming that the receptor decorates a subpopulation of early OPCs (Fig. 3b) in line with our already published results [20–22]. At the end of phase B, cells de-differentiated to multipotent and undifferentiated precursors characterized by strong GFAP immunoreactivity and branched morphology (Fig. 3c). Moreover, expression of both NG2 and GPR17 was dramatically downregulated compared to phase A (Fig. 3a). At the end of the last phase (phase C), that re-addresses GFAP⁺ undifferentiated precursors to all the three neural lineages, the majority of the cell population is represented by OPCs re-expressing NG2 and GPR17, whereas the number of GFAP⁺ cells was decreased compared to phase B (Fig. 3a, b). Interestingly, we also observed a trend to increase in the number of βIII-tub⁺ neuron-like cells (Fig. 3e; example picture in d), thus demonstrating that neurogenic protocol #1 succeeded in addressing a subpopulation of undifferentiated GFAP⁺ precursors towards a neuronal fate. In agreement with literature data, βIII-tub⁺ neuron-like cells showing a bipolar shape (Fig. 3d) never co-expressed either NG2 (see split fluorescence channels in Supplementary Fig. 1) or GPR17 (see also below).

We next characterized the progeny of OPCs grown under neurogenic protocol #2 after 1 day in DM or 3 days in SCM (see "Materials and methods"; Fig. 1). Also this protocol was able to unveil the multipotency of OPCs and to redirect NG2⁺ cells also towards a neuronal fate, as demonstrated by a significant increase in the number of βIII-tub⁺ neuron-like cells at the end of SCM phase compared to the end of phase DM (Fig. 4a). In parallel, the number of NG2⁺ cells decreased
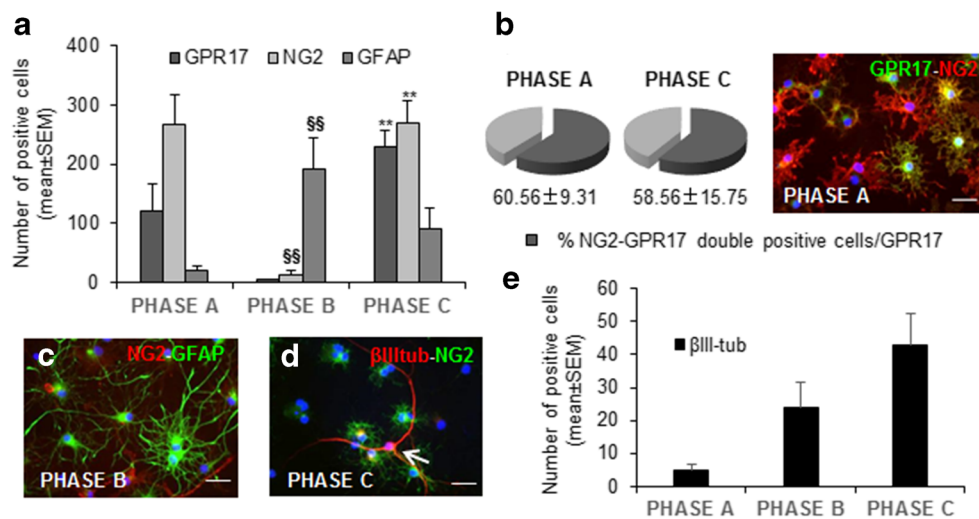
Fig. 3 Generation of βIII-tubulin⁺ cells by exposing NG2⁺ OPCs to neurogenic protocol #1. **a** Histograms showing the number of cells expressing the cell markers NG2, GFAP, and GPR17 at the end of the three phases of neurogenic protocol #1 (data are the mean of 3–10 coverslips from 2–5 independent experiments; §§$p < 0.01$ compared to phase A; **$p < 0.01$ compared to phase B; one-way ANOVA Bonferroni post hoc test). **b** Pie charts showing the percentage of NG2-GPR17 double-positive cells over the total number of GPR17⁺ cells at the end of phases A and C. Data are the mean of 4–7 coverslips from 2–3 independent experiments. A representative image showing the co-localization of GPR17 (green) and NG2 (red) at the end of phase A is shown on the right. Scale bar, 100 μm. **c, d** Representative images of the progeny of NG2⁺ cells at the end of phases B and C. Arrow in **d** indicates a typical βIII-tubulin⁺ (βIII-tub) neuroblast, which does not co-express NG2 (see split fluorescence channels in Supplementary Fig. 1). Scale bars, 100 μm. **e** The graph shows the number of βIII-tub⁺ cells at the end of each phase of neurogenic protocol #1
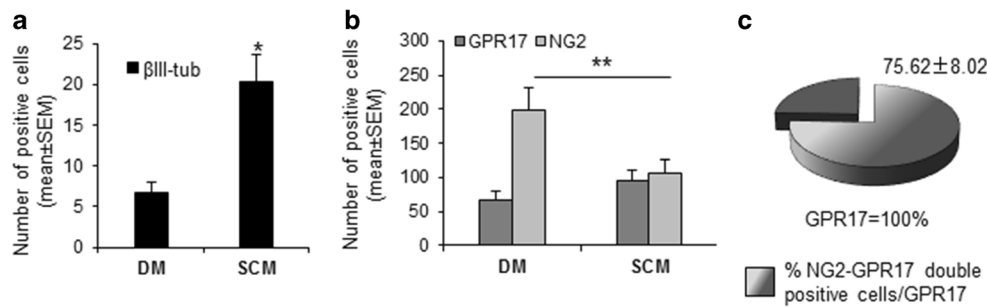
Fig. 4 In neurogenic protocol #2, incubation in stem cell medium (SCM) "per se" stimulates the generation of βIII-tubulin⁺ cells. **a**, **b** Histograms showing the number of βIII-tubulin⁺ (βIII-tub; **a**) or GPR17⁺ and NG2⁺ (**b**) cells at the end of phase DM or phase SCM (see Fig. 1b; *p < 0.05, and **p < 0.01 unpaired two-tailed Student's t test, data are the mean of 3–10 coverslips from 2–4 independent experiments). **c** Pie chart showing the percentage of GPR17⁺ cells co-expressing NG2 over the total population of GPR17⁺ cells at the end of phase DM. Data are the mean of 7 coverslips from 4 independent experiments

significantly when cells were shifted to SCM suggesting that at least a fraction of these cells were re-addressed towards a different fate. The number of GPR17-expressing cells was instead similar at the end of both phases (Fig. 4b), with the vast majority of the GPR17⁺ cells being NG2-expressing OPCs at the end of incubation in DM (Fig. 4c), as expected.

Valproic acid implements the neurogenic potential of OPCs and modulates the expression of GPR17

We then verified if and how exposure to VPA (500 μM) and to purinergic receptor ligands targeting GPR17 could modulate OPC multipotency and their commitment towards neurons with either neurogenic protocol. We chose a GPR17 antagonist (Cang; 10 μM) and a GPR17 agonist (UDP-glc; 100 μM [16, 20–23, 31–34]). In neurogenic protocol #1, cells were treated with the different pharmacological agents during phase C only, whereas in neurogenic protocol #2 cells were treated during both phase DM and SCM (see Fig. 1 and "Materials and methods" for details).

Exposure to VPA led to a significant increase in the total number of βIII-tub⁺ cells at the end of both neurogenic protocols, in parallel to a reduction in the number of GPR17⁺ cells (Fig. 5a). No differences were instead observed between control and VPA-treated cells in the expression of the OPC marker NG2 (data not shown).

To further confirm the neuronal commitment of OPCs, we performed Western blot analysis on cell lysates from CTR and VPA-treated cultures at the end of phase C (neurogenic protocol #1; Fig. 5b, left) or phase SCM (neurogenic protocol #2; Fig. 5b, right). Increase in βIII-tub and decrease in GPR17 expression were observed with either neurogenic protocol, thus fully confirming data from immunocytochemistry, and suggesting an attempt of OPCs to escape from their "classical" oligodendrocyte fate. In neurogenic protocol #2, we also investigated the expression of two neuronal synaptic proteins (namely, synaptic

vesicles 2, SV2, and synaptotagmin, syt). VPA induced a marked increase in SV2 expression compared to control cells, whereas Syt expression was unaffected (Fig. 5b, right). Taken together, our results confirm that VPA stimulates the differentiation of OPCs towards cells committed to neuronal differentiation, as suggested by the presence of synaptic vesicle proteins.

Interestingly, when primary rat OPCs were grown in standard differentiating conditions in the presence of VPA for 24 h, we also observed a reduction of GPR17 expression compared to control cultures (Fig. 5c), despite no changes in the generation of new neuron-like cells (see Fig. 2). This suggested us that VPA could have a direct action on GPR17 promoter, which is independent from its pro-neurogenic activity. Thus, to verify whether VPA directly influences GPR17 expression, we transfected the immortalized murine oligodendroglial Olineu cell line with a reporter construct in which an active portion of the GPR17 promoter had been previously cloned. In this reporter assay, VPA showed an inhibitory effect on GPR17 promoter activity compared to the empty vector (Fig. 5d). Overall, these data suggest that VPA can modify GPR17 expression in OPCs cells by directly acting on its promoter sequence.

Concerning the effects of the pharmacological modulation of GPR17, using neurogenic protocol #1, we observed a significant increase in the number of βIII-tub⁺ cells after exposure to Cang (Fig. 5e), whereas UDP-glc exerted no effect. Conversely, in neurogenic protocol #2, the number of βIII-tub-expressing cells seemed to be unaffected by either GPR17 ligand (Fig. 5f).

Exposure to either Cang or UDP-glc did not modify the number of GPR17-expressing cells in both neurogenic protocol #1 (Fig. 5e) and neurogenic protocol #2 (Fig. 5f). Thus, under the culturing conditions of neurogenic protocol #1, blockade of GPR17 receptor facilitates the neurogenic progression of OPCs, whereas receptor activation with UDP-glc exerts no effect.
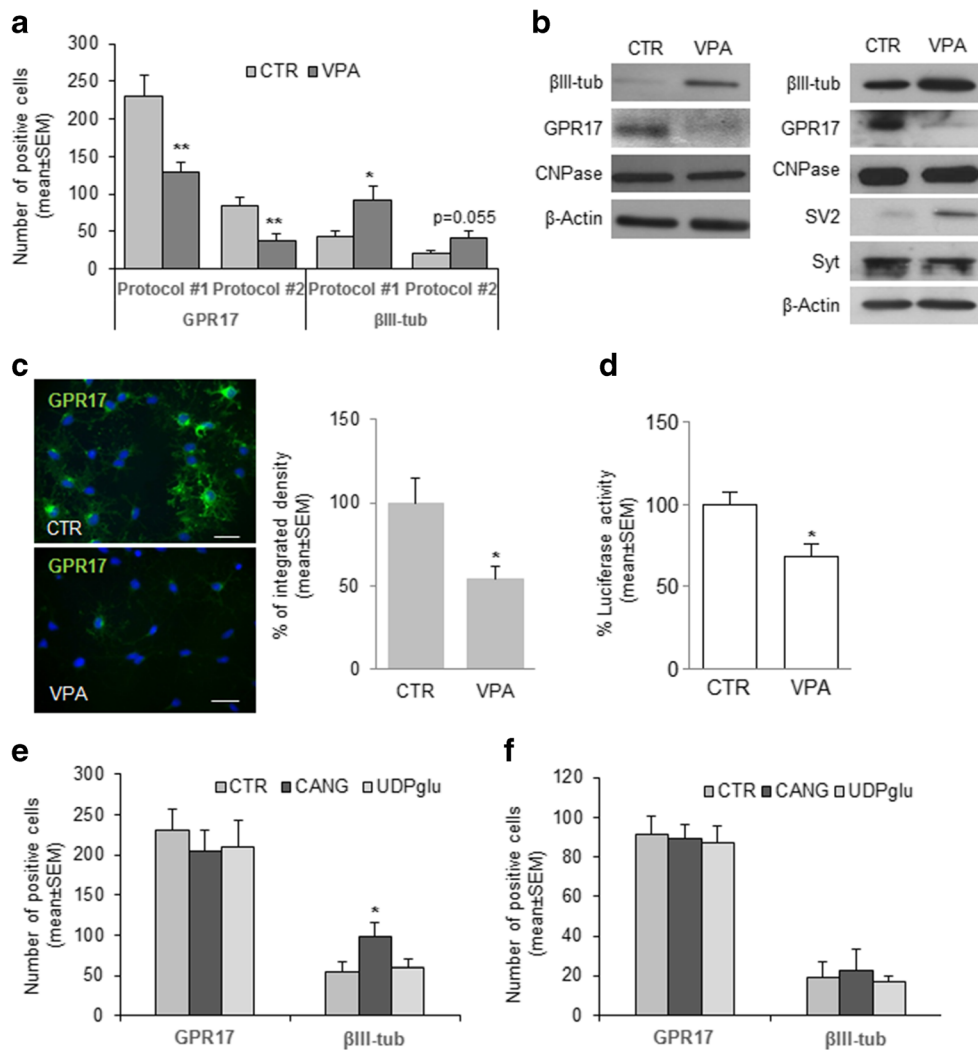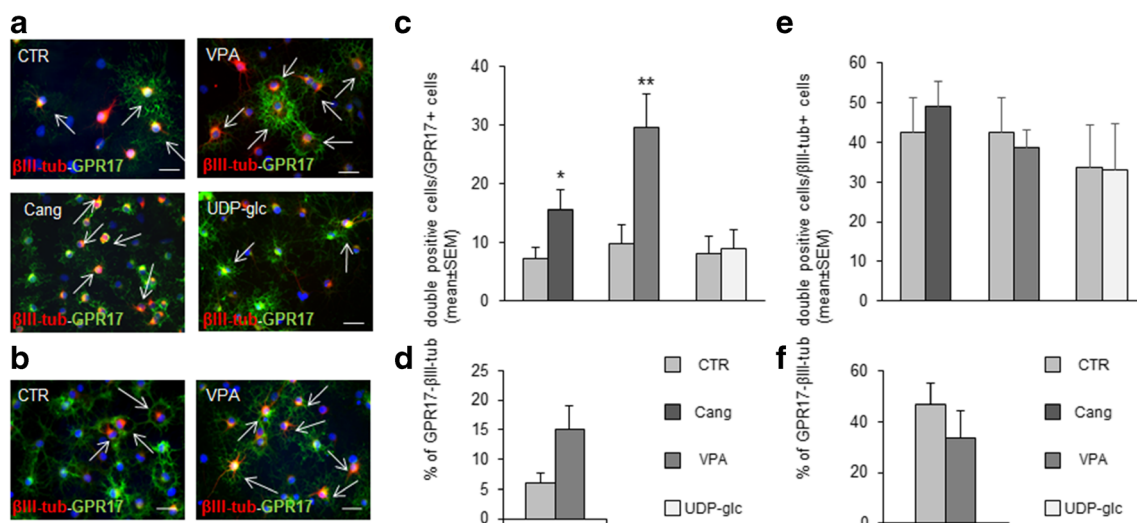
Fig. 5 In both neurogenic protocols, exposure to VPA fosters the generation of βIII-tubulin$^+$ neurons, in parallel with a decreased expression of GPR17. **a** Histograms showing the effect of the treatment with VPA on the number of GPR17 and βIII-tub$^+$ cells at the end of the two neurogenic protocols. Data are the mean of 12–20 coverslips from 5–10 independent experiments. *$p < 0.05$, **$p < 0.01$ unpaired two-tailed Student's t test. **b** Western blotting analysis of the expression of the neuronal proteins βIII-tub, synaptic vesicles 2 (*SV2*), and synaptotagmin (*syt*), of GPR17 and of the mature oligodendrocyte marker CNPase in cell cultures grown under control (*CTR*) condition or after VPA treatment in neurogenic protocol #1 (*left*) or #2 (*right*). β-Actin is shown as internal loading control. One representative experiment out of three is shown. **c** Representative images of GPR17 expression in OPC cultures grown in standard differentiating medium under control condition (*CTR*) or after a 24-h treatment with VPA. *Scale bars*, 100 μm. Histograms show the quantification of GPR17 expression measured as integrated density and expressed as arbitrary unit. Data are the mean of 3 coverslips/ condition from 1 representative experiment, and are shown as mean percentage ± SEM of CTR values set to 100 %. *$p < 0.05$ compared to CTR, unpaired two-tailed Student's t test. **d** Effect of VPA treatment on GPR17 promoter activity in Oli-neu cells. Luciferase activity was normalized to the cells transfected with the empty vector (*$p < 0.05$ unpaired two-tailed Students' t test). **e**, **f** Histograms showing the effect of the treatment with Cang or UDP-glc on the number of GPR17$^+$ and βIII-tub$^+$ cells at the end of the neurogenic protocol #1 (**e**) or #2 (**f**). Data are the mean of 9–7 coverslips from 3 independent experiments. *$p < 0.05$ unpaired, two-tailed Student's t test

Exposure to Cangrelor or VPA increases the percentage of GPR17$^+$ cells co-expressing βIII-tubulin

Two morphologically distinct groups of βIII-tub$^+$ cells were observed at the end of both neurogenic protocols: the first showed a typical bipolar neuroblast shape probably representing immature neurons (see above; arrow in Fig. 3d), whereas the second and more abundant type of cells still bore an OPC-like morphology (arrows in Fig. 6). As already mentioned above, in agreement with literature data, the former cell population never expressed GPR17. Conversely, in the latter cell population, a fraction of βIII-tub/GPR17 double-positive cells was detected already under CTR conditions at the end of phase C and of phase SCM (Fig. 6a, b). This is particularly interesting since no co-localization of GPR17 with neuronal markers has ever been

Fig. 6 VPA expands the percentage of GPR17⁺ cells also expressing βIII-tubulin in both neurogenic protocols. Representative pictures of cells grown according to neurogenic protocol #1 (**a**) or #2 (**b**) under the various experimental conditions. *Arrows* indicate "hybrid" GPR17/βIII-tub double-positive cells. *Scale bars*, 100 μm. **c** Histograms showing the percentage of GPR17/βIII-tub double-positive cells over the total number of GPR17⁺ cells at the end of neurogenic protocol #1. Results are the mean of 11–16 coverslips from 5–10 independent experiments. Neurogenic protocol #1: *$p < 0.05$ CTR vs. Cang; **$p < 0.01$ CTR vs.

VPA unpaired two-tailed Student's t test. A non-significant trend to increase by VPA was observed in neurogenic protocol #2 (**d**). **e, f** The percentage of GPR17/βIII-tub double-positive cells over the total number of βIII-tub⁺ cells is not modified by the different pharmacological treatments. In the case of neurogenic protocol #1, since the various drugs have been tested in separate experiments, the corresponding CTR values are reported for each pharmacological treatment (see panels **c** and **e**)

observed in vitro. The "hybrid" βIII-tub/GPR17 double-positive cell population was not detected in the early phases of either neurogenic protocol and may represent an intermediate precursor stage preceding the neuronal stage at which GPR17 is downregulated. Re-expression of GPR17 at this intermediate stage suggests that, in a subset of precursors and under specific neurogenic conditions, this receptor may contribute to drive precursor cells to a neuronal destiny.

On this premises, we analyzed the effects of the above-mentioned pro-neurogenic pharmacological treatments on the population of GPR17/βIII-tub double-positive cells under both neurogenic protocols. In neurogenic protocol #1, the percentage of GPR17/βIII-tub double-positive cells over the total cell population (evaluated by counting Hoechst-33258⁺ cell nuclei) was similar in control cultures or after exposure to UDP-glc (100 μM; control, 3.10 ± 1.29 %; UDPglc, 2.76 ± 0.89 %; 20 optical fields from 3 coverslips/condition), whereas it increased in cultures treated with either Cang (10 μM; control, 2.39 ± 0.62 %; Cang, 4.92 ± 1.00 %; $p < 0.05$ unpaired two-tailed Student's t test; 20 optical fields from 3 coverslips/condition) or VPA (500 μM; control, 2.39 ± 0.62 %; VPA, 5.38 ± 1.24 %; $p < 0.05$ unpaired two-tailed Student's t test; 20 optical fields from 3 coverslips/condition). An even more pronounced effect was observed when considering the percentage of GPR17/βIII-tub double-positive cells over the total number of GPR17⁺ cells, which in VPA- and Cang-

treated cultures was three times and about twice as high as in CTR cultures, respectively. No changes were observed with UDP-glc (Fig. 6a, c). With neurogenic protocol #2, at the end of phase SCM, a trend to increase in the percentage of GPR17/βIII-tub double-positive cells over the total number of GPR17⁺ cells was observed after exposure to VPA (Fig. 6b, d).

Conversely, if the population of GPR17/βIII-tub double-positive cells is expressed as percent of the total number of βIII-tub⁺ cells, no changes were observed upon the various pharmacological treatments (Fig. 6e, f). This further confirms that the observed effects are related to an action on GPR17, since either pharmacological treatment have no effect on the 60–70 % of βIII-tub⁺ cells that do not express the receptor.

Upon exposure to either VPA or Cang, we also observed a trend to a decrease in the percentage of NG2/GPR17 double-positive cells calculated on the total number of cells (CTR, 23.28 ± 10.85 %; VPA, 8.73 ± 2.11; and Cang, 8.49 ± 2.92 %, 20 optical fields from 3 coverslips/condition), thus confirming that a subset of OPCs is shifting its fate from oligodendrocytes to neurons. Nevertheless, GPR17/NG2 double-positive cells still represented the majority of the GPR17⁺ cell population (control, 58.56 ± 15.75 %; VPA, 45.62 ± 8.40; Cang, 55.10 ± 18.33 %, 20 optical fields from 3 coverslips/condition).

Taken together, our results suggest that VPA and, to a lesser extent, Cang are able to select a population of "hybrid" cells with an OPC morphology that starts acquiring neuronal markers and expresses GPR17.

Discussion

The main results of the present paper are as follows: (i) under specific neurogenic conditions in vitro, the transition from OPCs to cells of the three neural lineages is accompanied by appearance of an "hybrid" cell population, showing typical OPC morphology but expressing the neuronal marker βIII-tub; (ii) interestingly, this new cell population also co-expresses GPR17, already known to stimulate OPC differentiation towards fully myelinating oligodendrocytes; (iii) exposure of de-differentiated precursors to Cangr, a GPR17 antagonist, partially mimics the effects mediated by the anti-epileptic epigenetic agent VPA and accelerates cells' neurogenic conversion. These data show for the first time a co-localization between neuronal markers and GPR17 in vitro. Moreover, they extend the role of GPR17 in driving the fate of neural precursor cells, suggesting that, besides its involvement in oligodendrogenesis, this receptor may also instruct precursors to the neuronal lineage.

In vitro generation of βIII-tub$^+$ cells from NG2$^+$ OPCs

Our initial experiments demonstrate that a very low percentage of βIII-tub$^+$ cells is detected when OPCs are cultured in standard conditions, which are known to promote their maturation towards myelinating oligodendrocytes [20–23], even in the presence of the neurogenic agent VPA (Fig. 2; see also below). Besides, the trend to a reduction of their number over time in culture suggests that these cells could represent contaminating neurons surviving from the original mixed glial cell cultures.

Thus, we cultured NG2$^+$ OPCs according to a three-phase protocol, renamed here neurogenic protocol #1 [7], leading to their initial regression to an intermediate multipotent stage characterized by massive expression of GFAP (phase B) with no NG2 staining, which was then followed by GFAP down-regulation, re-appearance of NG2 and, most important, acquisition of the typical neuronal marker βIII-tub (phase C; Fig. 3). Interestingly, these phenotypic shifts were accompanied by significant changes of GPR17 expression, which was, as expected, highly expressed in NG2$^+$ cells but disappeared in the intermediate de-differentiated GFAP$^+$ cells (Fig. 3). This is in line with our previous in vivo and in vitro observations showing no co-localization of GPR17 with astrocytic markers [16, 23, 24] and demonstrating that GPR17 specifically decorates a subpopulation of early NG2$^+$ OPCs [20–22, 24]. A similar pattern of GPR17 co-localization with NG2 (Fig. 4) was observed with neurogenic protocol #2, which lacks the intermediate stage of cell de-differentiation to GFAP$^+$ precursors, but unveils OPC multipotency thanks to the exposure to a defined SCM known to support stem cell growth ([27, 37]; Fig. 1)

At the end of both protocols, the increased expression of the neuronal marker βIII-tub confirms that multipotency in vitro is an intrinsic feature of a subpopulation of NG2$^+$ OPCs. These results emphasize the notion that postnatal OPCs can no longer be considered as mere progenitors restricted to an oligodendroglial fate [38], but are indeed stem-like cells that can unveil their multipotency under specific conditions. This is also confirmed by the in vivo demonstration that, upon injury, adult parenchymal OPCs are reverted to an immature phenotype that more closely resembles that of neonatal OPCs [39].

Valproic acid fosters the neuronal commitment of OPCs and modulates GPR17 expression

As already shown in literature [40], our data show that the above-mentioned neurogenic shift is markedly implemented by exposure to VPA (500 μM), an anti-epileptic drug known to act as HDAC inhibitor ([41]; Fig. 5). In particular, VPA increased the expression of the neuronal marker βIII-tub, in parallel with a higher expression of synaptic vesicle protein 2 (SV2), an integral membrane protein expressed in synaptic vesicles [42, 43], suggesting commitment of OPCs towards neuroblasts that are acquiring proteins typically involved in synaptic transmission.

VPA treatment itself is not enough to induce OPCs to express βIII-tub under standard differentiating conditions (Fig. 2), but, nevertheless, VPA significantly reduced GPR17 expression even under these conditions (Fig. 5c), suggesting a direct effect of the drug on GPR17 expression. This effect is more evident when VPA is added to either neurogenic protocol (Fig. 5a, b), in line with the notion that inhibition of HDACs reduces the expression of genes driving oligodendrocyte specification [26–28]. Our reporter assay (Fig. 5d) also suggests that VPA inhibits GPR17 expression by likely regulating transcription factors that activate the GPR17 promoter, such as Olig1 or FoxO1 [18, 23, 44, 45], thus disrupting the balance between oligodendroglial commitment and stemcellness.

Despite being mostly expressed by NG2$^+$ cells, under neurogenic culturing conditions GPR17 was surprisingly expressed by a subset of βIII-tub$^+$ cells whose number was increased by exposure to VPA up to more than 25 % of the total number of GPR17-expressing cells (Fig. 6). This cell population showed the typical highly branched morphology of OPCs, thus probably representing a sort of "hybrid" of differentiation that is intermediate between the oligodendrocyte and neuronal fates. Thus, besides being a trigger for oligodendroglial differentiation [21], the P2Y-like receptor GPR17 may also be involved in the neurogenic specification of OPCs. This was already suggested by previous findings [46] that highlighted a role for GPR17 in the neuronal differentiation of PC12 cells, via modulation of the effects induced

by classical growth factors. It is worth mentioning that GPR17 expression was not observed on βIII-tub^{+} cells displaying typical neuroblast morphology (see arrow in Fig. 3d), suggesting that GPR17 expression in these precursors is transient and that the receptor is downregulated at later stages of neuronal differentiation. It may well be that GPR17 participates to neuronal specification during development, is turned down during adulthood, and is then re-activated under disease conditions, when endogenous reparative neurogenesis is switched on, as suggested by increased neuronal expression of GPR17 in the ischemic rat and mouse brain [16, 23], as well as in the injured spinal cord after a mechanical insult [24]. Alternatively, despite not being normally expressed in adult neurons, GPR17 may be involved in the formation of new neuronal cells in some specific brain areas where neurogenesis is preserved throughout adulthood, such as, for example, the hippocampus during learning and memory processes. In this respect, we have recently shown that GPR17 regulates the proliferation of a subpopulation of hippocampal DCX[+] neuronal progenitors involved in cognitive performances ([25]; see also below).

It has already been demonstrated that the systemic administration of VPA in rodents is associated with a reduction of oligodendrocyte generation and a corresponding increase of astrocytes and neurons [27]. Moreover, having been utilized in clinics as anti-epileptic [47], anti-migraine [48], and anti-maniac drug [49] for years, VPA profile in patients is rather well known, which could accelerate the translation of data to humans also for its use in other brain diseases. However, these very exciting perspectives must be confronted with the potential drawbacks linked to the high number of genes regulated through VPA-mediated mechanism [41].

Antagonism of GPR17 promotes the neurogenic transition of NG2^{+} precursors

Our current data show that increased neurogenesis was also observed by exposing cultures to the Cang, acting as a GPR17 antagonist, whereas no changes were detected with UDP-glc (Figs. 5, 6). Experiments were characterized by high variability, which is possibly linked to the use of heterogeneous primary cultures that are composed of non-synchronized cells at different stages of differentiation, and characterized by a different rate of proliferation, and by the application of complex, multistep neurogenic protocols. Thus, while the effect of VPA is very consistent due to its broad epigenetic mechanism, the variability observed upon GPR17 blockade can be explained with the transient expression of this membrane receptor during the whole differentiation program [19–22]. On the other hand, to explain the lack of significant effects by UDP-glc, cultured cells may themselves release endogenous GPR17 agonists that tonically regulate this receptor in vitro, thus making it difficult to unveil the effects mediated by exogenously added

compounds. Nevertheless, the present data showing implementation of the neurogenic fate by Cang are in strong agreement with our previous data on hippocampal GPR17 expressing DCX precursors, where receptor blockade with another antagonist, montelukast [16, 23], markedly increased the number of new mature neurons in parallel with increased animals' cognitive abilities [25].

In the present study, the most interesting and consistent results have been obtained with neurogenic protocol #1. This is possibly due to the fact that, at variance from protocol #2, this protocol involves the de-differentiation of NG2^{+} progenitors to GFAP^{+} multipotent cells (phase B), which are likely more prone to undergo epigenetic and pharmacological modulation towards a neuronal progeny. Nevertheless, the overall major length of protocol #1 compared to protocol #2 (13 vs. 6 days, respectively) poses technical problems for future experiments aimed at further fostering neurogenesis by prolonging cell exposure to either VPA or Cang. At the end of protocol #1, in fact, cells start showing signs of suffering and death (not shown) due to the length of time in culture. However, these results build up the background for further analysis, for the set-up of pharmacological/biotechnological approaches preventing excessive tissue degeneration while enhancing local reparative mechanisms. Importantly, GPR17 is a membrane receptor, thus amenable for activation/blockade by signals present in the local CNS milieu and for exogenous regulation by drugs, at variance from protocols based on genetic manipulations which are less prone to clinical exploitation, due to important drawbacks, such as the potential to cause tumor growth and gene mutations. In this respect, the pharmacological manipulation of endogenous cells still represents a safer and useful strategy for possible clinical applications. It is worth mentioning that the in vivo treatment of ischemic animals with Cang markedly prevented brain damage evolution [16, 23], suggesting a protective role against injury development. However, Cangr acts also as a potent antagonist at the P2Y$_{12}$ receptor subtype which has been shown to be highly expressed in the megakaryocyte/platelet lineage [50]. In this respect, FDA has recently approved it (June 2015) as an antiplatelet agent for intravenous application. The present findings unveiling the neurogenic properties of Cangr are particularly interesting in view of strategies aimed at repurposing this drug for additional neuroprotective uses that could be envisaged to speed up the development of urgently needed medicines for these patients.

Overall, based on the well-known role of purinergic signaling in regulating the synchronized proliferation, migration, differentiation, and death of embryonic and adult NPCs [51–54], our present results strengthen the evidence that the purinergic system crucially regulates neuronal progenitors

also in brain parenchyma, outside the well-known neurogenic niches. The pharmacological modulation of the purinergic system could therefore represent a promising and innovative approach to exploit the intrinsic ability of the adult brain to regenerate in acute and chronic neurodegenerative disorders.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The study has been approved by the Council of the Department of Pharmacological and Biomolecular Sciences of the Università degli Studi di Milano (Milan, Italy). Experiments have been performed in accordance with National and European regulations regarding the protection of animals used for experimental and other scientific purposes (D.L. 26_2014; 2010/63/UE), as well as following the Society for Neuroscience's policies on the Use of Animals and Humans in Neuroscience Research.

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