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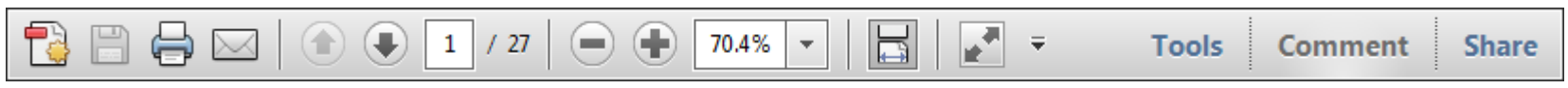

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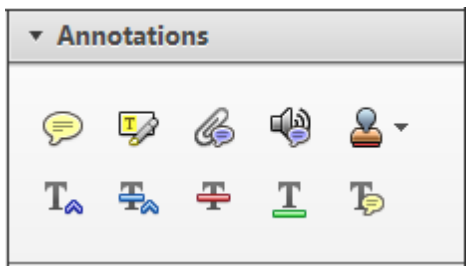

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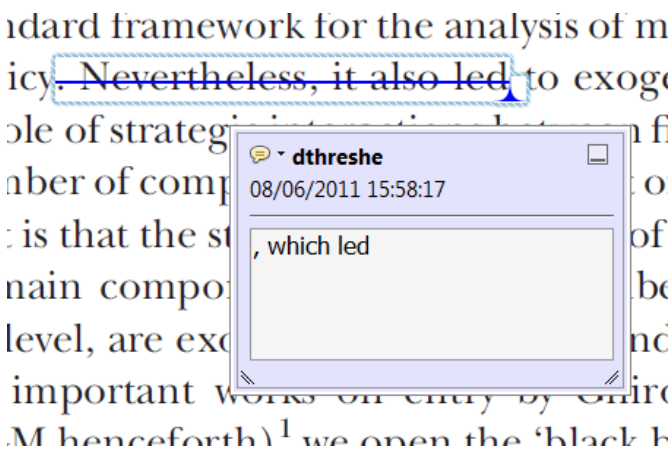

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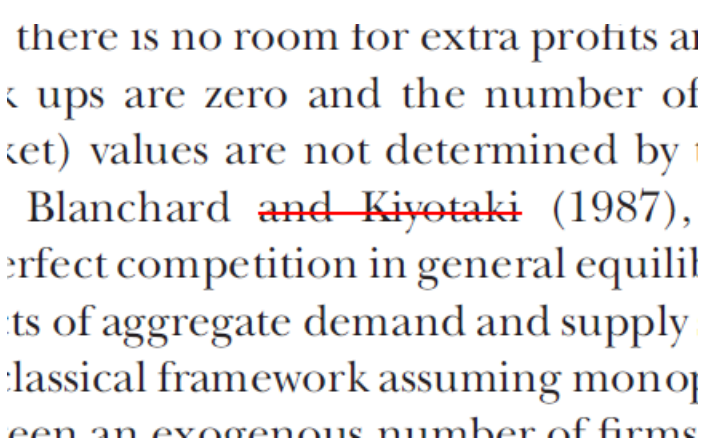

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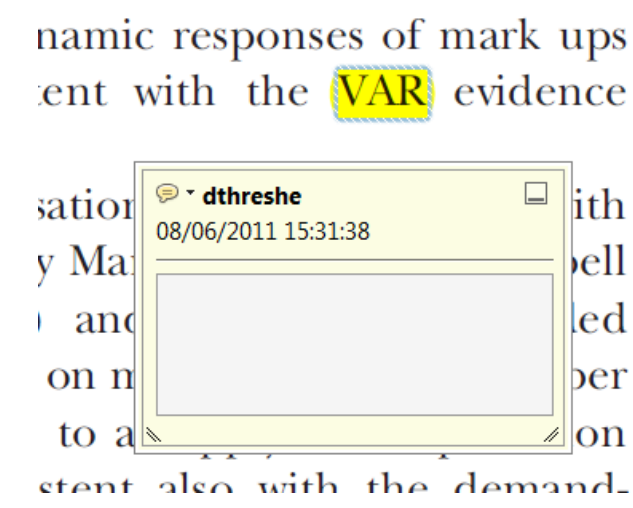

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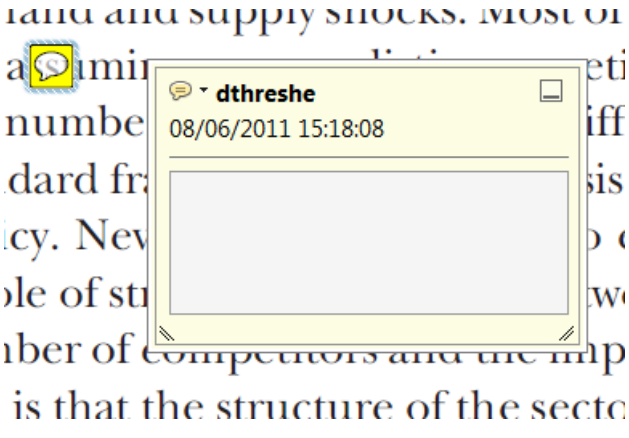

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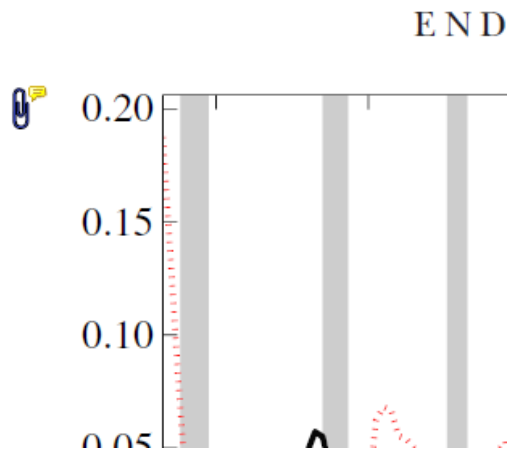

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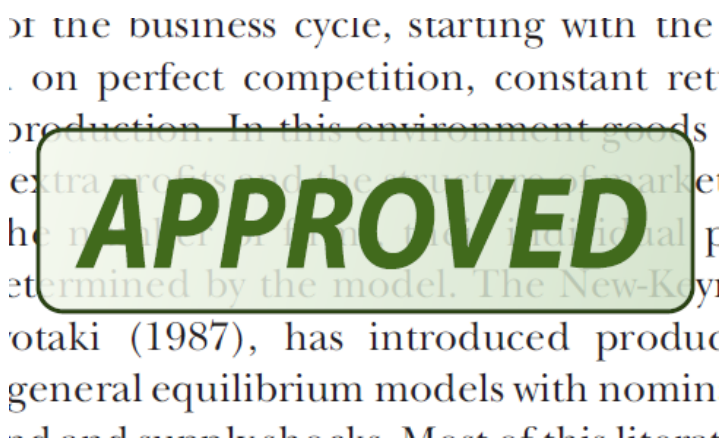

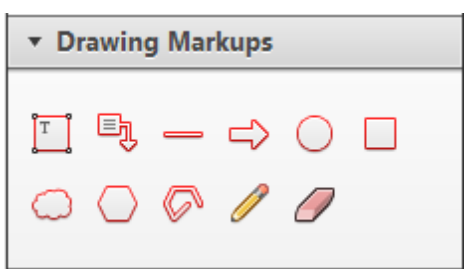

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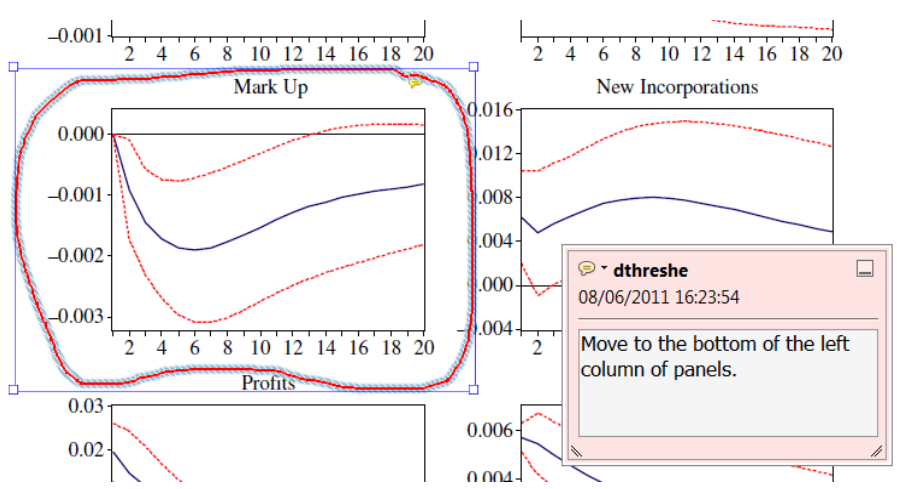

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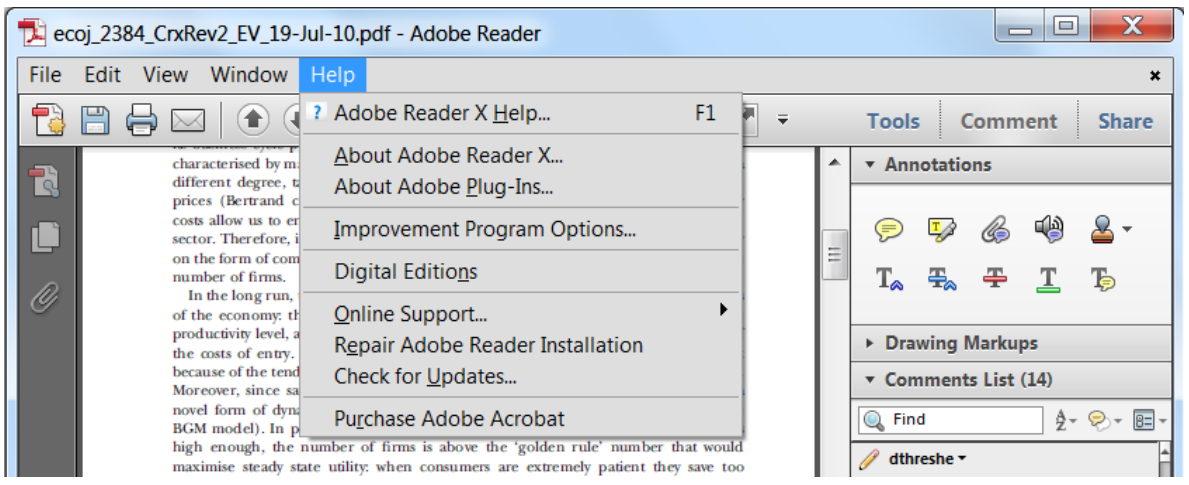

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Purines Regulate Adult Brain Subventricular Zone Cell Functions: Contribution of Reactive Astrocytes

Marta Boccazzi,[1] Chiara Rolando,[2] Maria P. Abbracchio,[1] Annalisa Buffo,[2] and Stefania Ceruti[1]

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Brain injuries modulate activation of neural stem cells (NSCs) in the adult brain. In pathological conditions, the concentrations of extracellular nucleotides (eNTs) raise several folds, contribute to reactive gliosis, and possibly directly affect subventricular zone (SVZ) cell functioning. Among eNTs and derived metabolites, the $P2Y_1$ receptor agonist ADP strongly promotes astrogliosis and might also influence SVZ progenitor activity. Here, we tested the ability of the stable $P2Y_1$ agonist adenosine 5′-O-(2-thiodiphosphate) (ADPβS) to control adult NSC functions both *in vitro* and *in vivo*, with a focus on the possible effects exerted by reactive astrocytes. In the absence of growth factors, ADPβS promoted proliferation and differentiation of SVZ progenitors. Moreover, ADPβS-activated astrocytes markedly changed the pattern of released cytokines and chemokines, and strongly modulated neurosphere-forming capacity of SVZ progenitors. Notably, a significant enhancement in proliferation was observed when SVZ cells, initially grown in the supernatant of astrocytes exposed to ADPβS, were shifted to normal medium. *In vivo*, ADPβS administration in the lateral ventricle of adult mice by osmotic minipumps caused diffused reactive astrogliosis, and a strong response of SVZ progenitors. Indeed, proliferation of glial fibrillary acidic protein-positive NSCs increased and led to a significant expansion of SVZ transit-amplifying progenitors and neuroblasts. Lineage tracing experiments performed in the GLAST::CreErt2; Rosa-YFP transgenic mice further demonstrated that ADPβS promoted proliferation of glutamate/aspartate transporter-positive progenitors and sustained their progression toward the generation of rapidly dividing progenitors. Altogether, the purinergic system crucially affects SVZ progenitor activities both directly and through the involvement of reactive astrocytes.

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Key words: $P2Y_1$ receptor, neurogenic niche, astrogliosis, adult neurogenesis

Introduction

Extracellular adenine (ATP and ADP) and uracil (UTP, UDP, and UDP-sugars) nucleotides (eNTs) act as cell-to-cell signals in the central nervous system (CNS) through the activation of seven ionotropic P2X (P2X1–7) and eight G protein-coupled P2Y receptor subtypes ($P2Y_{1,2,4,6,11,12,13,14}$; Abbracchio et al., 2006). eNTs are involved in embryonic development and act as co-transmitters in physiological processes (Ulrich et al., 2012). Recently, a role for eNTs in controlling the functions of stem cells in the subventricular zone (SVZ) of the adult brain has also emerged, although available data are conflicting.

ADP-responsive $P2Y_1$ receptor subtype ($P2Y_1R$) function has been tested *in vitro* on SVZ neural stem cells (NSCs) in the neurosphere assay. In this study, ADP or its stable analog 5′-O-(2-thiodiphosphate) (ADPβS) promoted the proliferation of SVZ cells, but only when growth factor (GF) concentrations were lowered to around 5 ng/ml (Mishra et al., 2006). Conversely, an antiproliferative effect of various

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Maria P. Abbracchio, Annalisa Buffo, and Stefania Ceruti contributed equally to this work

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eNTs, including ADPβS, was observed at standard GF concentrations (around 20 ng/ml; Stafford et al., 2007). Activation of the $P2Y_1R$ also promoted SVZ cell differentiation (Grimm et al., 2009) and migration (Grimm et al., 2010) *in vitro. In vivo*, the contribution of eNTs in controlling SVZ cell proliferation is poorly understood. In a recent study, ATP (which nonselectively activates all the P2X and some P2Y receptor subtypes) has been infused in the adult SVZ (Suyama et al., 2012). Upon ATP administration, the proliferation of transit-amplifying cells (type C cells) increased, whereas no effect was observed on proliferation of either type B stem cells or type A neuroblasts. A possible involvement of the $P2Y_1R$ was hypothesized based on the inhibitory effect on Mash1+ type C cell proliferation exerted by the administration of the $P2Y_1R$-selective antagonist 2′-deoxy-N^6-methyladenosine-3′,5′-bisphosphate (MRS2179) *per se* and by the reduced number of type C cells in $P2Y_1R$-KO mice (Suyama et al., 2012). In line with that, ATP secretion by astrocytes even at basal level can promote NSC proliferation in the adult hippocampus through $P2Y_1R$ activation (Cao et al., 2013). On contrary, activation of P2X7 receptor subtype with the stable ATP analog 3′-O-(4-benzoyl)benzoyl-ATP (Bz-ATP) induced a concentration-dependent decrease in NSC viability, suggesting a role for this receptor subtype in counterbalancing the proliferation of NSCs during pathological conditions, such as trauma, hypoxia/ischemia, and epilepsy, and/or in turning down this response at later times after injury (Delarasse et al., 2009; Messemer et al., 2013).

In this respect, in addition to their important physiological actions, eNTs modulate brain functions in pathological conditions, when their extracellular concentrations rise to micromolar levels (Abbracchio et al., 2006). Under these conditions, eNTs trigger and sustain reactive astrogliosis, the astrocytic reaction to brain trauma or ischemia (Abbracchio and Ceruti, 2006), whose protective/detrimental double-edged sword effect is still a matter of debate (Buffo et al., 2010). Activation of the ADP-responsive $P2Y_1R$ promotes astrogliosis and modulates astrocytic secretion of a plethora of factors, including cytokines/chemokines and GFs (Franke et al., 2012). These molecules might act as autocrine/paracrine signals on surrounding cells, including SVZ NSCs and progenitors.

On the basis of these premises, to investigate the role of purinergic signals in SVZ activation upon injury and to disclose possible effects specifically mediated by eNT-activated astrocytes, in this article we exposed SVZ cells and astrocytes to micromolar concentrations of ADPβS that recapitulate pathological conditions.

By means of *in vitro* and *in vivo* pharmacological manipulations combined with genetic fate mapping, we demonstrate that not only purinoceptors directly influence adult neurogenesis but that their activation on reactive astrocytes can further participate in controlling the activity of SVZ precursors in pathological conditions.

Materials and Methods

Neurosphere Assay

C57BL/6 adult mice were killed by decapitation. Cells were isolated from the SVZ and plated at low density (20,000 cells/ml), as described in literature (Pastrana et al., 2011; Rolando et al., 2012). Primary neurospheres were generated: (i) in neurosphere medium (containing 20 ng/ml EGF and bFGF each; Buffo et al., 2008), (ii) in neurosphere medium + ADPβS (50 μM) or in conditioned media derived from astrocytes cultured (iii) under control (ctr) condition, or (iv) in the presence of ADPβS (50 μM, Sigma-Aldrich, Milan, Italy; see below). This concentration of ADPβS was selected based on previous work showing that: (i) it activates astrocytes *in vitro* reproducing features of their response to lesion *in vivo* (Franke et al., 2012; Quintas et al., 2011) and (ii) it modulates adult stem cell functions (Grimm et al., 2009; Mishra et al., 2006; Stafford et al., 2007). In selected experiments, the $P2Y_1$-selective antagonist MRS2179 (50 μM, Sigma-Aldrich) was added at the time of SVZ cell plating to the neurosphere medium + ADPβS or to the conditioned medium derived from astrocytes cultured in the presence of ADPβS.

The number of generated neurospheres and their size were then evaluated. For each condition, neurospheres included in five randomly chosen optical fields at 10× magnification were analyzed under a Zeiss Axiovert 8400 microscope (Carl Zeiss, Milan, Italy) equipped with a CCD camera module. Neurosphere diameters were measured using the ImageJ software (Research Service Branch, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/). In selected experiments, primary neurospheres grown as indicated above were dissociated, and cells replated in control neurosphere medium irrespectively of their original culturing condition to generate secondary neurospheres. The number and size of secondary neurospheres were then analyzed after 2 days *in vitro* (DIV). Alternatively, to test cell proliferation and differentiation, 7-day-old control secondary neurospheres were allowed to adhere to the culturing substrate without dissociation, and were grown for 7 additional days in neurosphere medium without GFs and in the absence or presence of ADPβS (50 μM). Differentiation to astrocytes or neurons was evaluated by immunocytochemistry with the specific markers glial fibrillary acidic protein (GFAP) and βIII-tubulin (βIII-tub), respectively, whereas cell number was evaluated by staining nuclei with the Hoechst33258 dye (see below).

Immunocytochemistry, Image Processing, and Data Analysis

Adherent neurospheres were fixed in 4% paraformaldehyde and subjected to immunocytochemistry, as previously described (Lecca et al., 2008). Rabbit anti-GFAP (1:600, DAKO) and mouse anti-βIII-tub (1:1,000; Promega, Milan, Italy) primary antibodies were used (o/n at 4°C). Goat anti-rabbit or anti-mouse IgG conjugated with either Alexa Fluor® 488 or 555 (1:600; Life Technologies, Milan, Italy; 1 h at room temperature, RT) were utilized as secondary antibodies. Nuclear counterstaining was obtained by 20-min incubation with the Hoechst33258 dye (1:10,000 in phosphate-buffered saline, PBS). Coverslips were mounted in Dako Fluorescence Mounting Medium (Dako Italia, Milan, Italy) and analyzed under a Zeiss Axiovert 8400 microscope (Carl Zeiss) equipped with a CCD camera module.

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To evaluate the intensity of staining, fluorescent images were captured from 10 randomly chosen optical fields/coverslip. As a direct count of positive cells was technically not feasible, owing to their high number and the low fluorescence signal at the 10× magnification (which better allowed to gain a general view of the effect exerted by the pharmacological treatment; see Fig. 2), we performed a densitometric analysis of Hoechst33258, GFAP, and βIII-tub staining, after splitting the three fluorescence channels and converting the fluorescent signals to gray-scale values. The mean gray value for each optical field was then evaluated by the ImageJ software. Results represent the mean ± SEM of data from three coverslips derived from two independent experiments.

Primary Astrocytic Cultures

Primary astrocytic cortical cultures were obtained from P2 rats, as previously described (Ceruti et al., 2009). Cultures were grown for 10–12 days in Dulbecco's modified Eagle's medium with 20% fetal bovine serum (FBS; Euroclone, Milan, Italy). After removal of overlaying oligodendrocytes and microglia by vigorous shaking of flasks, astrocytes were kept in neurosphere medium (see above) in the absence (named "medium Astro ctr") or presence of ADPβS (50 μM; named "medium Astro ADPβS") for 3 additional days. Medium was collected, filtered, and utilized for the neurosphere assay or the cytokine array (see below).

Cytokine Array

The expression of cytokines, chemokines, and acute-phase inflammatory proteins was determined in neurosphere media and in the conditioned media from astrocytic cultures by means of the Proteome Profiler™ Rat Cytokine Array Panel A (R&D Systems, Italy). The array consists of nitrocellulose membranes spotted with antibodies directed against the following 29 proteins (alternative names are shown in parenthesis): CINC-1, CINC-2α/β, CINC-3, CNTF, Fractalkine, GM-CSF, sICAM-1 (CD54), IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-6, IL-10, IL-13, IL-17, IP-10 (CXCL10), LIX, L-Selectin, MIG (CXCL9), MIP-1α (CCL3), MIP-3α(CCL20), RANTES (CCL5), Thymus Chemokine (CXCL7), TIMP-1, TNF-α, and VEGF. The test was performed according to the manufacturer's instructions. Briefly, aliquots of the different culture media were mixed with a cocktail of biotinylated detection antibodies and then incubated overnight at 4°C on separate nitrocellulose membranes included in the array. This allowed the binding of any cytokine/detection antibody complex to its cognate immobilized capture antibody on the membrane. Following a wash to remove unbound material, streptavidin–horseradish peroxidase solution was added to the membrane for 30 min at room temperature. Detection of bound complexes was then performed by ECL (GE Healthcare) and autoradiography. The integrated optical density of each spot on the array was quantified by the Image J software.

Animals, Surgical Procedures, and In Vivo Treatments

Experiments were performed on C57BL/6 and GLAST::CreErt2; Rosa-YFP (Mori et al., 2006; Rolando et al., 2012) adult mice (2–4 months of age). The experimental plan was designed according to the guidelines of the NIH, the European Community Council (86/609/EEC), and the Italian laws for care and use of experimental animals (DL116/92). It was also approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin. Surgical procedures and perfusions were carried out under deep general anesthesia (ketamine, 100 mg/kg; Ketavet, Bayern, Leverkusen, Germany; xylazine, 5 mg/kg; Rompun; Bayer, Milan, Italy). GLAST::CreErt2; Rosa-YFP mice received tamoxifen dissolved in corn oil to induce Cre activity and YFP reporter expression (one administration of 5 mg each by oral gavage for 2 days) before starting the pharmacological treatment with the ADP analog. Osmotic minipumps (Alzet osmotic pumps 1007D) were implanted into the left cerebral ventricle (coordinates relative to bregma: anterior, 0; lateral, 1 mm; depth, 1.8 mm) to deliver ADPβS (100 μM in PBS; Sigma-Aldrich) or vehicle. The concentration of ADPβS was chosen based on literature data to reproduce the massive release of extracellular nucleotides (eNTs) that is observed following traumatic or ischemic brain injuries (Franke et al., 2012; Melani et al., 2005). Animals were killed 7 days after minipump implantation.

To analyze cell proliferation, animals received two i.p. injections of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich; 50 mg/kg in saline/day) on the last 3 days of ADPβS treatment. To facilitate discrimination of cell types in GLAST::-CreErt2; Rosa-YFP mice, we restricted the analysis to a small pool of cycling cells by injecting 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen; 50 mg/kg in saline i.p.; Ponti et al., 2013) to animals 2 h before killing. In these experiments, EdU was chosen instead of BrdU as its visualization follows a one-step procedure without DNA denaturation, and was therefore more convenient for triple immunostaining (see below).

Immunohistochemistry, Image Processing, and Data Analysis

For histological analysis, animals were anesthetized (see above) and transcardially perfused with 4% paraformaldehyde in PBS. Brains were collected, postfixed overnight, cryoprotected, cut coronally in 30-μm-thick slices, and stained according to the standard protocols (Rolando et al., 2012). Incubation with primary antibodies (anti-GFAP, 1:1,000, Dako; anti-doublecortin, DCX, 1:400, Santa Cruz Biotechnologies; anti-GFP, 1:700, Invitrogen; anti-BrdU, 1:250, Abcam; and anti-Mash1, 1:200, BD Pharmingen) was performed overnight at 4°C in PBS with 1.5% normal serum and 0.25% Triton-X 100. In the case of BrdU staining, slices were previously treated with 2 N HCl for 20 min at 37°C, followed by 10 min in 0.1 M borate buffer (pH 8.5). Sections were then exposed (2 h, RT) to secondary species-specific antibodies (all at 1:500 dilution) conjugated to Alexa Fluor® 488, 546, 649 (Life Technologies) or to Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA). Click-It™ EdU Cell Proliferation Assay Kit (Life Technologies) was utilized to detect EdU incorporation, according to the manufacturer's instructions. Nuclei were counterstained with the Hoechst33258 dye (1:10,000 in PBS; 20′ at RT; Life Technologies). Stained sections were mounted on microscope slides with Tris-glycerol supplemented with 10% Mowiol (Calbiochem, LaJolla, CA) and analyzed either by an E-800 Nikon microscope (Nikon, Melville, NY) equipped with a

color CCD Camera or by a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Quantifications were performed by ImageJ or Neurolucida (MicroBrightfield, Colchester, VT) software. Comparisons were made between hemispheres ipsilateral to the infusion site of control (ctr) and treated animals (unless differently stated). Analyses were performed on confocal images. Data are derived from a minimum of 6 up to 23 sections from three to four animals per experimental condition and/or time point. Results are expressed as either the absolute number of positive cells per section or per lateral wall (LW) length (which was similar in the analyzed samples, i.e., ctr mice, 283.62 ± 3.84 μm vs. ADPβS-treated animals, 295.93 ± 6.20 μm), as indicated in the figure legends and in the Results section.

Statistical Analysis

Data were analyzed using the GraphPad Prism5 software. Differences between experimental conditions were analyzed using either unpaired, two-tailed Student's *t*-test or one-way ANOVA followed by the Tukey correction. *P* value <0.05 was considered as significant.

Results

ADPβS Increases the Proliferation of SVZ Precursors and Their Lineage Progression In Vitro

To examine the ability of ADPβS to modulate proliferation and multipotency of SVZ cells, we performed the neurosphere assay in the presence of standard concentrations of both bFGF and EGF (20 ng/ml each; see Materials and Methods). After 7 DIV, the number and size of primary neurospheres were analyzed. Exposure to 50 μM ADPβS increased the neurosphere-forming capacity of SVZ cells, as indicated by the expanded number of spheres (Fig. 1A,A′,B). Yet, ADPβS stimulation determined the generation of neurospheres with a reduced size compared with controls (Fig. 1A,A′,C). Furthermore, the total number of cells yielded after the dissociation of neurospheres at 7 DIV decreased upon treatment (1,476,562 ± 271,023 in ctr cultures vs. 686,094 ± 168,364 after exposure to ADPβS; $P < 0.05$ Student's *t*-test). These findings are in line with an antiproliferative effect of ADPβS in the presence of regular GFs concentration (see Introduction). Co-exposure to the selective $P2Y_1R$ antagonist MRS2179 (50 μM) completely abrogated ADPβS-induced effects on both neurosphere-forming capacity and proliferation (Fig. 1A″,B,C), thus demonstrating that the ADP analog is selectively activating the $P2Y_1R$.

To assess the effects of $P2Y_1R$ activation in the absence of GFs and to examine the outcome of ADPβS exposure on the lineage progression of SVZ cells, 7-day-old ctr secondary neurospheres were plated without dissociation and grown for 7 additional days in a medium without GFs (see Materials and Methods) in the absence or presence of ADPβS. The expression of the neuronal and astrocytic markers βIII-tub and GFAP, respectively, and the mean area of adhering neurospheres were evaluated by immunocytochemistry, in parallel with the staining of cell nuclei by the Hoechst33258 dye (Fig. 2A,A′). Exposure to 50 μM ADPβS led to a moderate, but significant increase in neurosphere area (121.11% ± 17.38% of ctr neurosphere area set to 100.00% ± 12.89%; 19–21 optical fields from three coverslips per condition; $P < 0.05$, Student's *t*-test). A much higher increase in the mean fluorescence value for Hoechst33258 staining was detected after exposure to the purine analog (Fig. 2B,B′,E), meaning a higher number of cell nuclei per area.

Interestingly, immunoreactivity for both GFAP and βIII-tub clearly increased upon exposure to ADPβS (Fig. 2C–D′). Although the increase in GFAP immunoreactivity was proportional to the increased cell number (compare Fig. 2E and 2F), βIII-tub staining in ADPβS-treated cultures exceeded the labeling in ctr condition by twofold (Fig. 2G), indicating a prominent effect of ADPβS on neuroblast production. Therefore, *in vitro* results indicate that ADPβS promotes neurosphere formation and stimulates the generation of neurons and astrocytes.

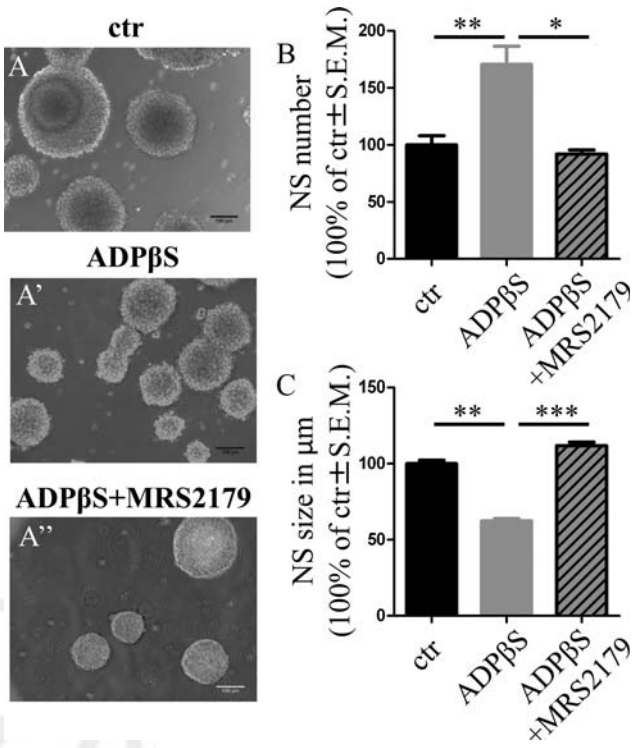

FIGURE 1: ADPβS modulates neurosphere formation from the SVZ *in vitro*. (A, A″) Representative micrographs showing 7-day-old primary neurospheres (NS) generated under ctr condition (A) in the presence of ADPβS alone (A′) or in combination with MRS2179 (A″). Quantification of the number (B) and size (C) of primary neurospheres generated under the various experimental conditions (three replicates from two independent experiments; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, one-way ANOVA followed by Tukey *post hoc* analysis). Scale bars: 100 μm.

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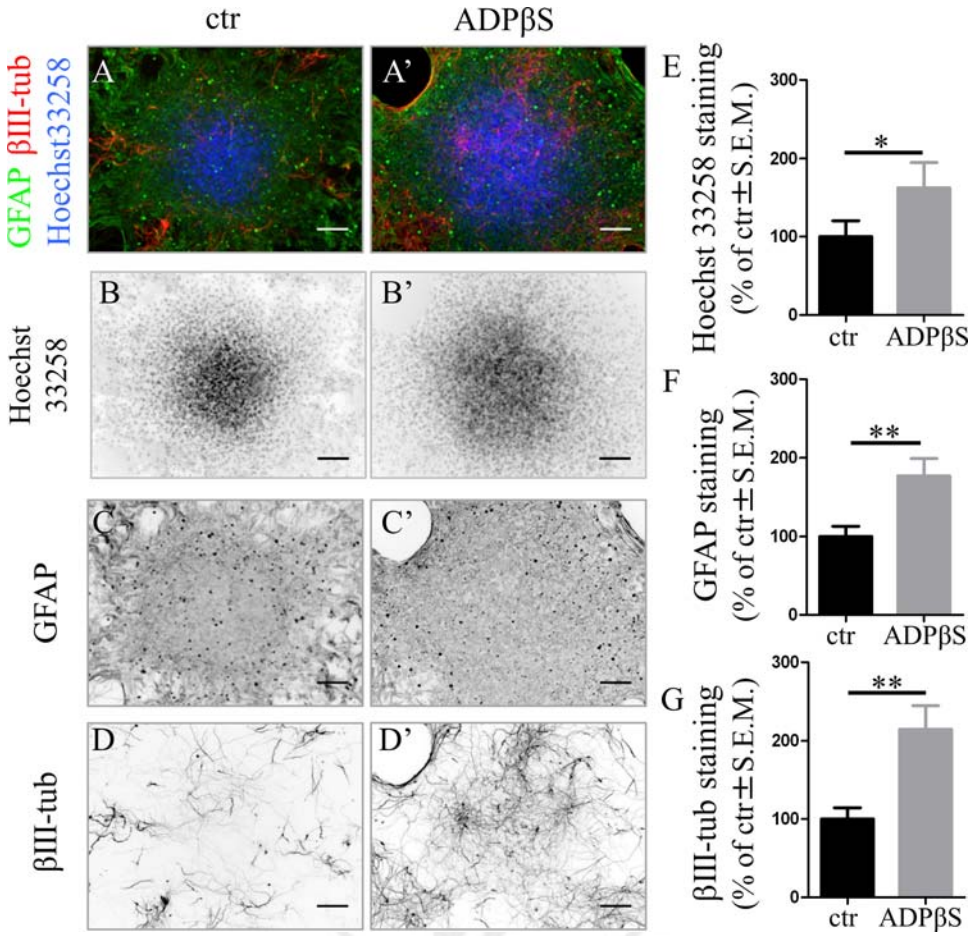

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FIGURE 2: ADPβS stimulates neurosphere differentiation and proliferation. (A, A′) Immunofluorescence staining for βIII-tub (red) and GFAP (green) of adherent undissociated neurospheres (NS) cultured in differentiating conditions in ctr medium (A) or in the presence of ADPβS (A′). Nuclei were counterstained with the Hoechst33258 dye (blue). (B–D′) the green, red, and blue fluorescent channels in A and A′ were separated and converted to gray scale for densitometric analysis, shown in (E) for Hoechst33258, in (F) for GFAP, and in (G) for βIII-tub. Data are shown as the mean percentage ± SEM of ctr values set to 100% (19–21 optical fields from at least three coverslips per condition, *$P < 0.05$; **$P < 0.01$, unpaired Student's *t*-test). Scale bars: 100 μm.

Astrocytes Exposed to ADPβS Influence the Generation of Neurospheres from SVZ Cells In Vitro

Solid evidence demonstrates that exposure of astrocytes to nucleotides in general, and to ADPβS in particular, induces reactive astrogliosis (Abbracchio and Ceruti, 2006; Franke et al., 2012). Reactive astrocytes release several mediators (see Introduction), which could influence the functionality of surrounding cells such as SVZ progenitors. To unveil the possible effects of ADPβS-activated astroglia on neural progenitors, we assessed whether astrocyte-conditioned media could modify neurosphere formation by SVZ cells. To this aim, we exposed primary astrocyte cultures to conventional neurosphere medium (with GFs, see Materials and Methods) with or without 50 μM ADPβS (see Fig. 3A for experimental design). Both culture media were then collected after 3 DIV and utilized to generate neurospheres as described above. When SVZ progenitors were grown in medium from control (Astro ctr) or ADPβS-treated astrocytes (Astro ADPβS), no differences were found in either the number (number of generated neurospheres: 17.67 ± 2.56 in Astro ctr medium vs. 14.00 ± 3.36 in Astro ADPβS medium, $P > 0.05$, Student's *t*-test) or the size of primary neurospheres at 7 DIV (Fig. 3B). To exclude that the Astro ADPβS medium contained: (i) residual ADPβS or (ii) adenine nucleotides released by activated astrocytes, which could influence SVZ cell properties, the $P2Y_1$ antagonist MRS2179 was added to Astro ADPβS medium at the time of neurosphere generation. No significant differences were found in the neurospheres formed in Astro ADPβS medium or in Astro ADPβS + MRS2179 (Fig. 3B), thus ruling out a role for eNTs in the conditioned medium from reactive astrocytes.

However, despite the generation of a similar number of primary neurospheres in Astro ctr and Astro ADPβS media compared with standard (formerly indicated as ctr) neurosphere media (not shown), a significantly lower number of cells was yielded from their dissociation. In fact, the total number of cells was 1,476,563 ± 271,023 from ctr neurospheres vs. 157,400 ± 36,603 and 132,500 ± 46,199 from

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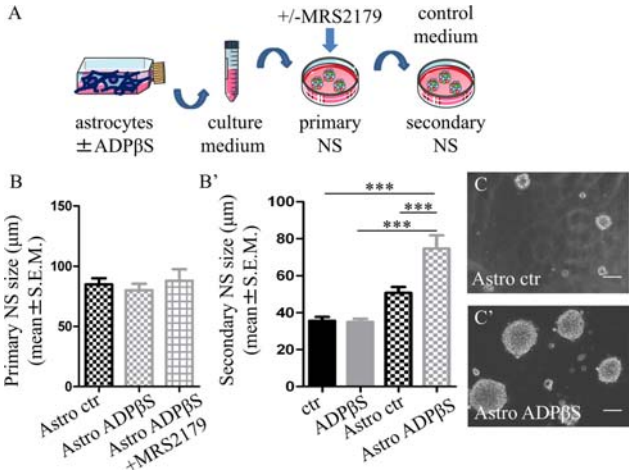

FIGURE 3: ADPβS-treated astrocytes regulate the generation of secondary neurospheres from SVZ precursors *in vitro*. (A) Schematic representation of the experimental design (see text for details). (B) Evaluation of the size of primary neurospheres (NS) generated in the conditioned medium from ctr or ADPβS-treated astrocytes. In selected experiments, the selective P2Y$_1$R antagonist MRS2179 was added to Astro ADPβS medium at the time of seeding of SVZ cells. (B′) Evaluation of the size of secondary neurospheres generated in fresh ctr medium from cells obtained by the dissociation of primary neurospheres grown under the various experimental conditions (five coverslips from two independent experiments; ***$P < 0.001$, one-way ANOVA followed by Tukey *post hoc* analysis). Representative micrographs are shown in C, C′. Scale bars: 100 μm. The cartoon was produced thanks to "Servier Medical Art" (www.servier.com).

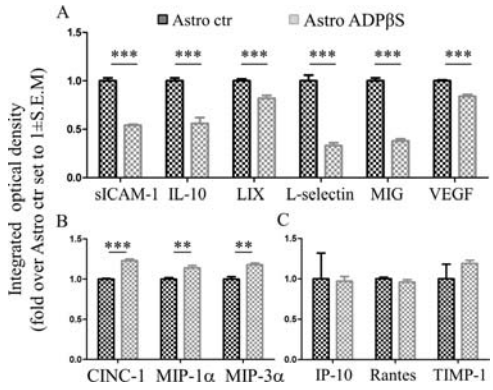

FIGURE 4: ADPβS modulates the release of various signaling molecules from astrocytes *in vitro*. Relative expression of various signaling molecules in conditioned medium derived from ctr or ADPβS-treated astrocytes, as determined by an antibody array (see Materials and Methods for details). A and B show molecules whose extracellular concentration decreased or increased after ADPβS exposure, respectively. C shows unchanged molecules. Results are the mean of two independent analyses ($P < 0.01$, ***$P < 0.001$, unpaired Student's *t*-test).**

neurospheres grown in Astro ctr and Astro ADPβS media, respectively ($P < 0.001$ ctr neurospheres vs. Astro ctr and ctr neurospheres vs. Astro ADPβS, Student's *t*-test). Of further interest, these numbers were also lower than those obtained with exposure to ADPβS alone (686,094 ± 168,364; $P < 0.05$ ADPβS vs. Astro ctr and ADPβS vs. Astro ADPβS, Student's *t*-test), suggesting a specific negative astrocyte-mediated effect on precursor proliferation.

Primary neurospheres were then dissociated and replated in ctr neurosphere medium, irrespectively of their initial growing condition, to generate secondary neurospheres (Fig. 3A). Surprisingly, fully formed secondary neurospheres were already generated at 2 DIV from cells derived from primary neurospheres grown in Astro ADPβS medium (Fig. 3B′,C′). Conversely, very small cell clusters were visible in cultures derived from primary neurospheres grown in Astro ctr medium (Fig. 3B′, compare Fig. 3C with 3C′) or in ctr and ADPβS medium (Fig. 3B′). Numbers of neurospheres were instead similar in all the tested conditions (not shown). These findings suggest that ADPβS specifically acts on astrocytes by promoting the release of mediator(s) capable to "prime" SVZ precursors inducing them to proliferate. However, this proliferative effect is possibly concomitantly inhibited by other astrocyte-derived factor(s), so that only after their removal from the culture medium the proliferation of SVZ cells is intensively boosted. To identify possible mediators released in the astrocyte-conditioned medium and responsible for the above observed effects, we took advantage of the Proteome Profiler^{TM} Antibody Array. This approach allows the simultaneous detection of 29 cytokines and chemokines in the tested culture media (see Materials and Methods). In ctr and ADPβS media, the concentrations of all the analyzed molecules were below the detection limit of the array (not shown). In both Astro ctr and Astro ADPβS media, CINC-2α/β, CINC-3, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-6, IL-13, IL-17, CNTF, GM-CSF, TNF-α, Fractalkine, Thymus Chemokine, and IFN-γ were not detected, but 12 of the 29 tested molecules were instead found (Fig. 4). Of these, only IP-10, Rantes, and TIMP-1 concentrations did not change between the two experimental conditions (Fig. 4C). Instead, exposure to the nucleotide analog either significantly reduced or increased astrocytic release of the majority of the other detected mediators. Specifically, IL-10 and some molecules involved in cell adhesion (like sICAM-1 or L-selectin) were decreased, whereas chemoattractant chemokines and some other cytokines involved in cell migration (CINC-1, MIP-1α, and MIP-3α) were increased (Fig. 4A,B). F4

Thus, a combination of various secreted mediators, whose release by astrocytes is modulated by ADPβS stimulation, might account for the observed effects on SVZ cell properties.

The P2Y$_1$R Agonist ADPβS Activates Both Niche and Parenchymal Astrocytes and Increases the Proliferation of SVZ Precursors In Vivo

Next, we aimed to translate *in vivo* our *in vitro* data, and to address the role of P2Y$_1$R in the regulation of adult neurogenesis in conditions comparable to those observed after brain

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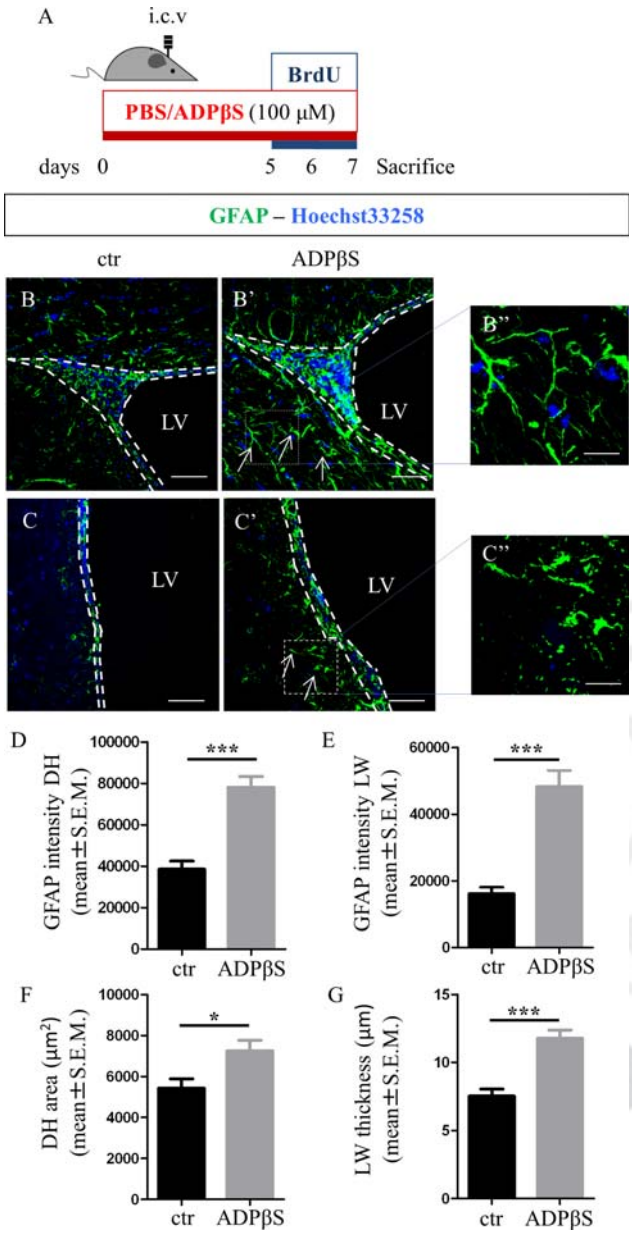

FIGURE 5: A 7-day-long infusion of ADPβS increases GFAP immunostaining in the SVZ and induces reactive astrogliosis in brain parenchyma. (A) Experimental protocol (see Materials and Methods for details). (B, B′, C, C′) Representative images of GFAP staining (green) in the dorsal horn (DH) and lateral wall (LW) of ctr (B, C) and ADPβS-treated animals (B′, C′). Significant reactive astrogliosis induced by the nucleotide analog was evident in brain parenchyma (arrows in B′, C′ and insets B″, C″). (D, E) Quantification of GFAP immunostaining in the DH (D) and LW (E). Values represent the mean fluorescence intensity of GFAP staining per section in arbitrary units. (F, G) Quantification of the DH area (F) and of LW thickness (G), as delimited by the dashed lines in (B, B′) and (C, C′), respectively. (*$P < 0.001$, Student's t-test). Scale bars: 50 μm in B–C′ and 150 μm in B″, C″. LV, lateral ventricle.**

injury. Thus, ADPβS (100 μM) was chronically infused for 1 week into the cerebral ventricles of wild-type mice and cell proliferation was monitored by BrdU administration during the last 3 days (see Materials and Methods and Fig. 5A). Incorporation of the thymidine analog was then evaluated by immunohistochemistry together with the expression of cell-specific markers for SVZ populations and parenchymal astrocytes (i.e., GFAP for astrocytes and type B precursor cells, Mash1 for type C transit-amplifying cells, and DCX for type A neuroblasts; Doetsch et al., 1997; Kim et al., 2011).

Notably, ADPβS infusion caused a significant GFAP upregulation in both astrocytes of the brain parenchyma (arrows in Fig. 5B′,C′ and insets B″,C″), confirming our *in vitro* and literature data demonstrating a role for eNTs in reactive astrogliosis (Franke et al., 2012), and in the neurogenic areas of the dorsal horn (DH) and LW (Fig. 5B,B′ and C,C′; quantification in D and E, respectively), where GFAP also labels precursor cells. In parallel, a significant expansion of DH area (Fig. 5F) and a thickening of the LW (Fig. 5G) were observed, confirming a specific effect of ADPβS on GFAP+ type B cells (i.e., stem cells and niche astrocytes).

In line with these observations, ADPβS stimulation promoted cell proliferation in the SVZ. A parallel significant increase in BrdU incorporation was detected in the LW (Fig. 6A,B). Namely, we observed an expansion of Mash1+ type C transit-amplifying cells (Fig. 6C,D), which also incorporated more BrdU (Fig. 6E). Moreover, the total number of DCX+ neuroblasts and the fraction of DCX/BrdU double-positive cells also significantly increased after ADPβS exposure (Fig. 6G–I). However, in either cell populations, the percentage of proliferating cells (i.e., the number of cells double-positive for both BrdU and the cell population-specific marker/the total number of cells for each cell population × 100) was not increased by the nucleotide analog (Fig. 6F,J). This suggests that ADPβS does not enhance the proliferation of either one (or both) cell populations, but rather acts by stimulating the proliferation of their parent precursors or by globally overactivating all SVZ populations. Similar results were obtained in the DH (not shown).

ADPβS Promotes the Generation of Rapidly Dividing Cells from GLAST-Expressing Stem Cells

Next, to verify whether ADPβS can foster the transition of stem-like precursors toward transit-amplifying cells and neuroblasts, we took advantage of GLAST::CreERT2; Rosa-YFP transgenic mice. In these animals, cells expressing GLAST (i.e., SVZ type B cells and parenchymal astrocytes) and their progeny are permanently labeled by the fluorescent protein YFP upon Tamoxifen (Tam in Fig. 7A) administration. Animals chronically received either PBS or 100 μM ADPβS in the lateral ventricle for 1 week (Fig. 7A). Two hours before sacrifice, animals received a single administration of the

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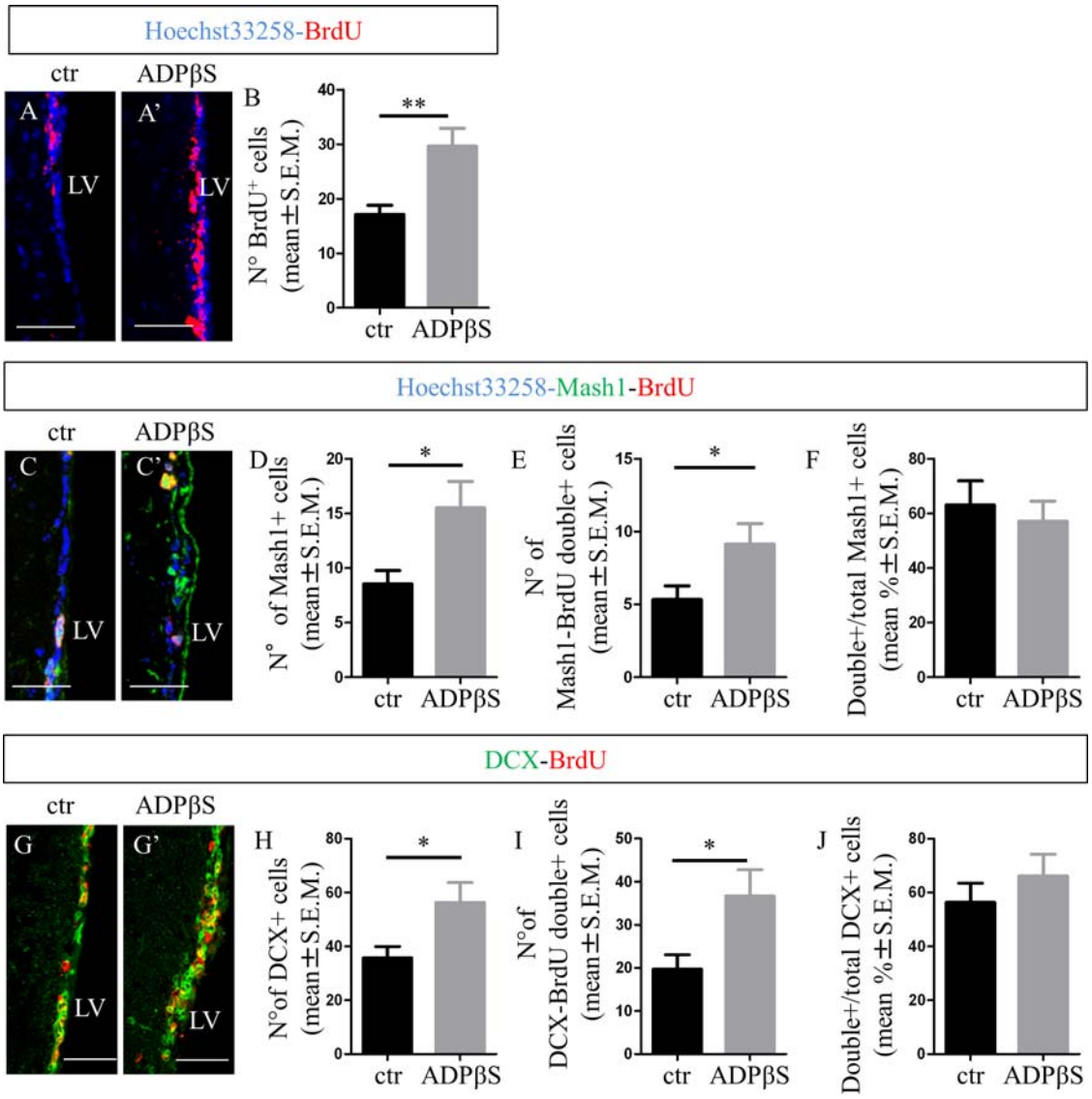

FIGURE 6: A 7-day-long infusion of ADPβS stimulates the proliferation of SVZ cells and increases the number of Mash1+ and DCX+ cells. (A, A′) Representative images of the LW from ctr (A) or ADPβS-treated animals (A′) immunolabeled for BrdU (red). (B) Quantification of the total number of BrdU+ cells in the LW ($P < 0.01$, unpaired Student's *t*-test). (C, C′) Representative images of the LW from ctr (C) or ADPβS-treated animals (C′) immunolabeled with anti-Mash1 (green) and anti-BrdU (red). (D–F) Quantification of Mash1+ (D), of Mash1-BrdU double-positive cells (E), and of the percentage of proliferating cells in the Mash1+ population (F) (*$P < 0.05$, unpaired Student's *t*-test). (G, G′) Immunofluorescence images of DCX (green) and BrdU (red) staining in the LW of ctr (G) or ADPβS-treated animals (G′). (H–J) Quantification of the total number of DCX+ (H), of DCX-BrdU double-positive cells (I), and of the percentage of proliferating cells in the DCX+ population (J) (*$P < 0.05$, unpaired Student's *t*-test). Scale bars: 50 μm. In all images nuclei were counterstained with the Hoechst33258 dye (blue). LV, lateral ventricle.**

thymidine analog EdU to label actively dividing cells. In line with previous data, we detected an increased incorporation of EdU upon exposure to the purine analog (Fig. 7B,C). Moreover, the number of YFP/EdU double-positive cells was increased twofold (Fig. 7D), indicating that ADPβS treatment expanded a population of GLAST+ cell-derived progenitors in active proliferation. Although this population was for the most composed of GFAP-negative progenitors (Fig. 7E), likely including Mash1+ and DCX+ cells (see above), exposure to ADPβS also resulted in a twofold increase of actively cycling YFP/EdU double-positive progenitors also expressing GFAP along the ventricle (Fig. 7F,G). Furthermore, a small but significant increase in the total number of YFP/GFAP double-positive cells was detected after treatment (Fig. 7H). These data indicate that ADPβS activates GFAP+ precursors in the SVZ with a consequent expansion of their progeny.

Discussion

The main finding of our study is that the purinergic system promotes proliferation and lineage progression of SVZ precursors

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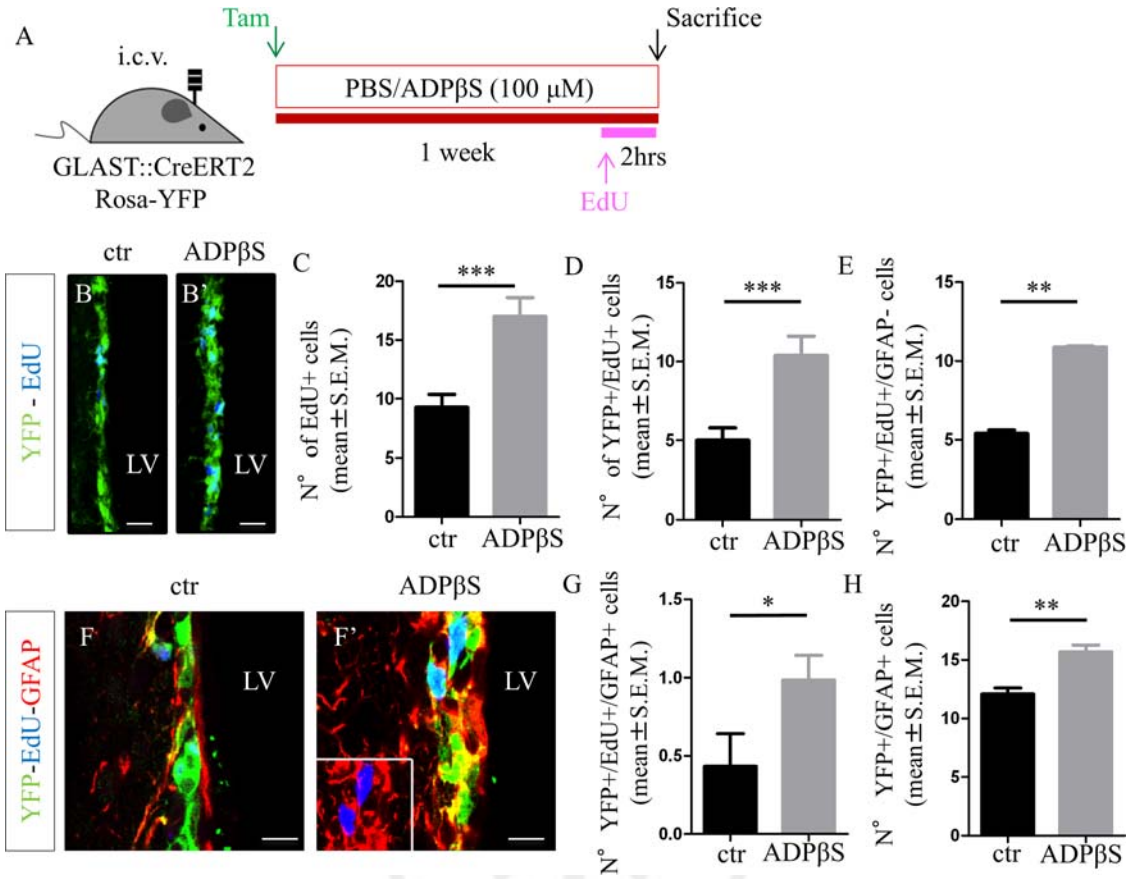

FIGURE 7: ADPβS promotes the proliferation of rapidly dividing transit-amplifying cells and GFAP+ precursor cells (A) Experimental design (see text for details). (B, B′) Immunofluorescence images of EdU incorporation (blue) and YFP (green) staining in the LW of ctr (B) and ADPβS-treated animals (B′). (C, D) Quantification of the number of LW cells incorporating EdU (C) and also positive for YFP (D). (E) Quantification of YFP-EdU double-positive cells, which do not express GFAP. (F, F′) Triple immunostaining (i.e., YFP, green; EdU, blue; GFAP, red) in the LW of ctr (F) and ADPβS-treated animals (F′). Inset shows in detail GFAP/EdU double staining in the triple labeled cells placed at the upper right corner of F′. (G, H) Quantification of YFP-EdU double-positive cells also expressing GFAP (G) and of GFAP-YFP double-positive cells (H) ($P < 0.01$, ***$P < 0.001$; unpaired Student's *t*-test). Scale bars: 15 μm in B, B′ and 10 μm in F, F′. LV, lateral ventricle.**

in vitro and *in vivo*, both directly acting on progenitors, and indirectly through the involvement of reactive astrocytes. Thus, eNTs act as local extrinsic factors regulating NSC functions, particularly following traumatic or hypoxic events, when progenitors are in contact with high concentrations of ATP and its metabolites (i.e., ADP, sequentially followed by adenosine), owing to the high activity of ATP-metabolizing ectonucleotidases in neurogenic brain areas (Lin et al., 2007). The high micromolar concentrations of extracellular ATP and its metabolites under these pathological conditions (Abbracchio et al., 2006) also contribute to induction and modulation of reactive astrogliosis (Abbracchio and Ceruti, 2006). Thus, to clarify the role of purinergic system on NSC behavior during brain injury, we used experimental settings that reproduce *in vivo* and *in vitro* a pathological environment. To this aim, we administered ADPβS, a stable analog of the ATP metabolite ADP, instead of ATP, which is highly instable and undergoes fast hydrolysis (Dunwiddie et al., 1997).

NSCs grown *in vitro* in the presence of GFs, like EGF and bFGF, and ADPβS display increased neurosphere-forming capacity, partially in accordance with previous work, where $P2Y_1R$ activation brings to increased neurosphere formation (Mishra et al., 2006; Stafford et al., 2007). Moreover, ADPβS promotes the formation of neurospheres with a decreased diameter, suggesting that the interaction of purinergic signals with EGF and bFGF reduces cell proliferation, as already reported (Stafford et al., 2007). Notably, $P2Y_1R$ activation may cause different effects on NSC function that appear dependent on culturing condition (i.e., GFs concentration and neurosphere passages) and may also reflect changes over time in the intrinsic properties of cultured progenitors (Mishra et al., 2006; Stafford et al., 2007). Moreover, exposure to ADPβS under prodifferentiative *in vitro* conditions (i.e., by removing GFs from culturing media) leads to stimulation of neuronal and, to a lesser extent, astrocytic commitment of NSCs. Altogether, *in vitro* findings show that

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purinergic signaling can positively regulate neurosphere formation and neurogenesis under defined culturing conditions.

The above i*n vitro* effects were fully confirmed *in vivo* by ADPβS i.c.v. infusion at micromolar concentrations (100 μM), with a significant upregulation of GFAP expression in both brain parenchyma and SVZ, showing an ADPβS-mediated activation of astrocytes not only at parenchymal sites (Franke et al., 2004, 2009) but also in the neurogenic area (see also below). Moreover, BrdU incorporation showed that $P2Y_1R$ activation stimulates cell proliferation, consistent with a global stimulation of SVZ activity. In line with these results, lineage analysis in GLAST::CreERT2; Rosa-YFP mice exposed to ADPβS showed an increase of both all GFAP/YFP double-positive cells and of the actively cycling fraction. Furthermore, the immediate progeny of GLAST+ precursors (i.e., Mash1+ and DCX+ cells) was also expanded. Altogether, these findings point to an effect of activation of $P2Y_1R$ on GFAP+ type B cells leading to an expansion of Mash1+ and DCX+ populations.

By administering the natural agonist ATP for 3 days to mice, Suyama et al. recently observed an increase in the number of both rapidly dividing BrdU+ cells and Mash1+ type C cells in the SVZ with no effects on GFAP+ type B cells (Suyama et al., 2012). Differences in the agonist chosen (ADPβS vs. ATP) and in protocols of administration (with a 7-day-long infusion in our study) could explain the differences in the results obtained. Moreover, apart from its fast rate of hydrolysis *in vivo* with a half-life of hundreds of milliseconds (Dunwiddie et al., 1997), ATP can activate a wide variety of purinergic receptors, spanning from the seven P2X ionic channels to some P2Y subtypes (mainly the $P2Y_2$ and $P2Y_4$ receptors; Fischer and Krugel, 2007). In addition to this, ATP is rapidly degraded first to ADP (which stimulates the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors; Abbracchio et al., 2006) and finally to adenosine, which activates the four types of P1 adenosine receptors. Thus, the different effects exerted by ATP may be related to a more complex pattern of receptor activation with respect to ADPβS that is known as a selective agonist at the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptor subtypes and to be relatively resistant to ectonucleotidase hydrolysis (Ralevic and Burnstock, 1998).

Literature data suggest that the $P2Y_1R$ receptor is mediating the effects of eNTs on both precursor cells and reactive astrocytes (Franke et al., 2001; Suyama et al., 2012). This was confirmed by the complete reversal of ADPβS-mediated effects exerted by the $P2Y_1R$ receptor antagonist MRS2179 in our *in vitro* experiments. Moreover, published data and RT-PCR analysis showed that all the three SVZ cell populations and primary astrocytes expressed the mRNA for this receptor subtype (not shown; Fumagalli et al., 2003). Therefore, we are confident that all the observed effects are related to the activation of the $P2Y_1R$. Nevertheless, at present, we cannot completely exclude the contribution of the two additional ADP-sensitive P2Y receptors (i.e., the $P2Y_{12}$ and $P2Y_{13}$), whose mRNA was also detected in SVZ cells.

Our work also assessed the role of astrocyte reactivity on SVZ cell functions. Reactive astrogliosis virtually occurs in all injuries and pathologies and therefore likely affects NSC activity and neurogenesis, although knowledge on this issue is limited. Therefore, we exposed SVZ cells to conditioned media from astrocytic cultures grown under ctr conditions or in the presence of ADPβS, and found a negative effect of both media on the expansion of primary neurospheres. Previous *in vitro* data showed a stimulatory action of cultured astrocytes on the proliferation and neuronal differentiation of NSCs based on both contact-mediated and secreted factors (Barkho et al., 2006; Lim and Alvarez-Buylla, 1999; Song et al., 2002). In another study, reactive astrocytes activated *in vitro* by a mechanical insult were found to promote astrogliogenesis from NSCs via released factors with no changes in progenitor proliferation compared to nonactivated cultures (Faijerson et al., 2006). Our findings unveil a previously unknown inhibitory effect of astrocyte-derived soluble factors on SVZ progenitor activities, in line with an inhibitory action *in vivo* of reactive astrocytes on stem cell maintenance and neuron production (Buffo et al., 2010; Larsson et al., 2004).

Astrocytes can release ATP already under basal conditions, which in turn influences NSC function in the adult hippocampus via $P2Y_1R$ (Cao et al., 2013). However, this does not seem the case in our experimental setting, because addition of the $P2Y_1R$ antagonist MRS2179 did not revert the observed effects.

A complex pattern of soluble mediators is probably involved in astrocyte-mediated inhibition of NSC functions. Specifically, cytokines/chemokines and adhesion factors have been proposed to control these cells, although their role is far from being understood (Christie and Turnley, 2013). In this respect, some interesting hints come from our analysis of cytokine/chemokine/GFs content of conditioned media. IP-10 (also known as CXCL10), TIMP-1, and Rantes were absent in normal neurosphere medium and highly expressed in ctr and ADPβS astrocyte medium at comparable levels, and could therefore account, at least partially, for the observed generalized inhibitory effect of astrocyte-conditioned media on NSCs. So far, these signals have been implicated in the control of differentiation or motility of immature neurons or astrocytes, respectively (Lee et al., 2011; Park et al., 2009). A few studies reported a role for these cues in NSCs specification (e.g., IP-10 in combination with other cytokines; Barkho et al., 2006) and increased motility upon damage (TIMP-1; Ben-Hur et al., 2006; Rantes; Guan et al., 2008). However, their function in SVZ cells remains to be thoroughly

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investigated. Cues with promigratory effects on SVZ cells (CINC-1 and MIP-1α; Gordon et al., 2009) were also detected in astrocyte media, with a slight increase after ADPβS stimulation. By altering cell-to-cell interactions required to sustain NSC activity, these signals, in combination with others and with GFs present in our experimental setting, could lead to a generalized inhibition of NSC properties.

In addition to this, a number of astrocyte-secreted molecules, which are known to stimulate stem cell properties also outside the brain (such as VEGF, IL-10, and LIX; Calvo et al., 2011; Choong et al., 2004; Perez-Asensio et al., 2013; Yang et al., 2009), and to be involved in cell adhesion (such as sICAM-1, L-selectin, and MIG), were downregulated upon exposure of astrocytes to ADPβS. Conversely, the astrocytic release of molecules with chemoattractive and migration properties was increased by ADPβS. Globally, these data are in line with the prodifferentiative role exerted by the ADP analog.

Interestingly, a significant enhancement in neurosphere formation was detected when secondary neurospheres were generated in standard medium from cells derived from primary neurospheres grown in the supernatant of astrocytes exposed to ADPβS. This suggests that removal of inhibitory mediators triggered upon exposure to the purine analog boosted the proliferation of SVZ cells during secondary neurosphere culturing. Alternatively, restoration of the stimulatory factors/pathways attenuated by the supernatant of ADPβS-exposed astrocytes could trigger this proliferative compensative response in secondary neurospheres.

We are currently working on this hypothesis, which suggests that during acute phases after damage eNTs-mediated reactive astrogliosis does not directly affect the proliferation of SVZ progenitors, but rather modifies their intrinsic properties, leading to a boost toward neurogenesis at more later stages when the concentrations of the various mediators decline. *In vivo*, the situation is likely to be more complicated owing to the presence of reactive microglia, whose functions and properties can be profoundly affected by eNTs (Fumagalli et al., 2011).

In conclusion, our results represent a significant step forward the full understanding of the role of eNTs in controlling SVZ cell functions under pathological conditions and help building the substrate for therapeutic strategies that limit brain damage and promote tissue regeneration.

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