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Title	RELEASE OF SOLUBLE AND VESICULAR PURINE NUCLEOSIDE PHOSPHORYLASE FROM RAT ASTROCYTES AND MICROGLIA INDUCED BY PRO-INFLAMMATORY STIMULATION WITH EXTRACELLULAR ATP VIA P2X7 RECEPTORS
Article type	Research Paper

Abstract

Purine nucleoside phosphorylase (PNP), a crucial enzyme in purine metabolism which converts ribonucleosides into purine bases, has mainly been found inside glial cells. Since we recently demonstrated that PNP is released from rat C6 glioma cells, we then wondered whether this occurs in normal brain cells. Using rat primary cultures of microglia, astrocytes and cerebellar granule neurons, we found that in basal condition all these cells constitutively released a metabolically active PNP with Km values very similar to those measured in C6 glioma cells. However, the enzyme expression/release was greater in microglia or astrocytes that in neurons. Moreover, we exposed primary brain cell cultures to pro-inflammatory agents such as lipopolysaccharide (LPS) or ATP alone or in combination. LPS alone caused an increased interleukin-1β (IL-1β) secretion mainly from microglia and no modification in the PNP release, even from neurons in which it enhanced cell death. In contrast, ATP administered alone to glial cells at high micromolar concentrations significantly stimulated the release of PNP within 1 h, an effect not modified by LPS presence, whereas IL-1β secretion was stimulated by ATP only in cells primed for 2 h with LPS. In both cases ATP effect was mediated by P2X7 receptor (P2X7R), since it was mimicked by cell exposure to Bz-ATP, an agonist of P2X7R, and blocked by cell pre-treatment with the P2X7R antagonist A438079. Interestingly, ATP-induced PNP release from glial cells partly occurred through the secretion of lysosomal vesicles in the extracellular medium. Thus, during inflammatory cerebral events PNP secretion promoted by extracellular ATP accumulation might concur to control extracellular purine signals. Further studies could elucidate whether, in these conditions, a consensual activity of enzymes downstream of PNP in the purine metabolic cascade avoids accumulation of extracellular purine bases that might concur to brain injury by unusual formation of reactive oxygen species.

Keywords	neural cell cultures; extracellular purine nucleoside phosphorylase (PNP); P2X7 receptor; mechanism of release; lysosomal vesicles.

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Data will be made available on request

Dear Editor,

we are re-submitting the article in which we demonstrated that cultures of rat brain cells, in particular astrocytes and microglia, release purine nucleoside phosphorylase (PNP), usually known as a cytosolic enzyme involved in the metabolism/salvage pathway of adenine- and guanine-based purines. We demonstrated that lipopolysaccharide (LPS), able to cause the release of interleukin 1 beta (IL-1β) from glial cells did not affect PNP release. In contrast, high concentrations of extracellular ATP, that may be present in inflamed brain tissue, enhanced IL-1β release from the same cells primed with 2h LPS, whereas PNP release was increased even in the absence of LPS priming. Finally, ATP effects were likely mediated *via* P2X7 receptors and the ATP-induced PNP secretion in part occurred through a lysosomal pathway, which is active in different pathological brain conditions (brain ischemia, traumatic injury or and seizures) and is used for cytokine secretion from cells.

Following the reviewers' suggestions, we entirely revised our manuscript and figures and we do hope that now it is may be considered acceptable for publication on this Journal.

Best regards,

Renata Ciccarelli

Corresponding Author

The Authors want to thank the reviewers and the Editor for their observations which they tried to fulfill, as reported below.

Comments from the editors and reviewers:
This study is well planned and reports interesting new data. There are some missing information and controls that need to be added and several details that need to be trimmed. The comments are tendered in order of appearance in the text.

Abstract
-(p.6) 'an excessive formation of extracellular purine bases may concur to brain injury by unusual formation of reactive oxygen species'. Was it shown that hypoxanthine levels increased? Was it considered that uric acid is an effective antioxidant and the levels of uric acid correlate inversely with the incidence of neurodegenerative disorders such as Parkinson's disease?
R: We agree with the reviewer and changed the sentence that was only a speculation on possible dangerous effects due to the eventual accumulation of hypoxanthine consequent to an increase in the amount/activity of extracellular PNP. Moreover, we know that these effects may be mitigated by (more or less rapid) transformation of hypoxanthine in uric acid.

Introduction
-(p.8) 'the metabolic activity of this enzyme outside the cells would be important to assure an homeostatic control of the purinergic system'. Could this be explained? Does PNP inhibition affect the levels of adenosine or ATP?
R: As widely known at intracellular levels, there is a finely tuned metabolism of purines that allows to adequately restore the intracellular pool of nucleotides avoiding their lack or accumulation, the former leading to a decrease in energy supply or cell duplication rate, the latter inducing cell death as pointed out for instance in lymphocytes lacking PNP. At the extracellular level, the coordinated activities of the enzymes is necessary to modulate the interaction of active purine compounds with their own receptors and also to guarantee an adequate re-uptake of nucleosides and bases to reconstitute the intracellular pool of purines. As for the second question, we recently demonstrated (Giuliani et al., J. Neurochem. 2016) that hypoxic conditions in rat C6 glioma cells lead to a lower presence/activity of PNP, that in turn causes a longer permanence, in the extracellular medium, not only of neuro-protective compounds (GUO and INO), whose levels likely contribute to inhibit the activity of Xanthine Oxidase and, as a consequence, to decrease the amount of XAN and Uric Acid, but also of ATP and GTP. We tried to include this explanation in the text of the current manuscript.

-(p.9) 'we aimed to better elucidate the localization and function of PNP in the brain'. Elucidating the localization of PNP in the brain would require histological techniques. Cultured cells, no matter how informative and useful, cannot assess brain histology.

R: we agree with the reviewer and reworked adequately the sentence.

-(p.9) ATP as a 'ubiquitous substance that is released from all neural cells and achieves high extracellular concentrations during acute and inflammatory brain damages'. The seminal work of Di Virgilio was clearly the first to document the role of ATP as a danger signal, but only a recent review established ATP was recently labeled as a danger signal in the brain (Rodrigues et al., 2015, Front Cell Neurosci 9:148).
R: we included the suggested reference containing a comprehensive review of the current literature to support the role of ATP as danger signal.

Methods
-(p.14) 'media were desalted'. I have always been mystified by this terminology. All proteins are salts. If samples are desalted, the Western analysis should be blank...
R: As correctly suggested by the reviewer, we explained better the methodology used for the preparation of conditioned media to be used in western blot analysis, by changing the sentence "media were desalted" with "while inorganic salts were removed from media, which were further concentrated by using Amicon YM-3 (Millipore)".

-(p.16) '15-min non-linear gradient'. A gradient implies the use of at least two eluents. None is mentioned. This reviewer cannot have any idea of the type and quality of the analysis.
R: The HPLC method used in this study for determining PNP activity has been widely described by the article (Giuliani et al., 2016) cited at the beginning of the subsection *2.6. Measurement of PNP activity,* thus in this subsection it was only briefly described without reporting all the chromatographic conditions, but of course this may be confusing or unclear to the reader. Thus to improve reading comprehension and as suggested by the Reviewer, the sentence has been changed as follows:
Briefly, separation was obtained on a Phenomenex Kinetex pentafluorophenyl analytical column (5µm pore size, 100 Å particle size, 250×4.6 mm; Phenomenex INC, Castelmaggiore, Bologna, Italy) at 35◦C, using a mobile phase composed of 0.1% (v/v) formic acid in water (solution A) and methanol (solution B). Separation was carried out with a 15 min non-linear gradient elution while the flow rate (of the mobile phase) was set at 1ml/min.

-(p.16) 'the non-fluorescent compounds were spectro-photometrically monitored at 254nm'. Maybe 'other non-fluorescent purines' since several cellular compounds do not absorb light at 254 nm.
R: The Reviewer is right, in this sentence we mean that, except for GUO and GUA, the other purine compounds, if present, were monitored by UV detection at 254 nm; so the sentence has been changed as follows:

Autofluorescent GUO and GUA were monitored at an excitation wavelength of 260 nm and an emission wavelength of 375 nm while eventual other purine compounds present in the eluate were spectrophotometrically monitored at 254nm.

-*(p.17) 'The glial specificity of siRNA transfection is due to the fact that mature CGNs are not transfected by LipofectAMINE200'. In at least three collaborating labs, the efficiency of lipofectamine transfection was always circa 5% in cultured microglia cells. The efficiency of transfection of each of the 3 types of culture needs to be made explicit.*
R: We had already performed measures of transfection efficacy in our conditions, therefore we added the following sentence in the methods paragraph: "The vector pmaxGFP® (Amaxa, Cologne, Germany) was used to test transfection efficiency in both microglia and CGNs primary cultures, as previously described (Peña-Altamira et al., 2014; Polazzi et al., 2013), showing that when using LIPOFECTAMINE-2000, while GFP-positive neurons are almost undetectable in CGN cultures, microglial GFP-positive cells are about 17±2% respect to total Hoechst stained cells."

Results
-*(p.19) 'in all neural cells'. I don't understand this terminology. Glial cells are brain cells, but not neural cells.*
R: although we found that the adjective "neural" is often used also to indicate glial cells, we changed "neural" with "brain" that seems more appropriate.

-*(p.19) 'PNP mRNA was silenced'. What was the efficiency of transfection? This is critical to determine the usefulness of this approach in populations of cells.*
R: As previously stated, we described the methodology to test the efficiency transfection in the methods, where we also indicated the transfection efficacy. Furthermore, we clarify the concept by changing the sentence "PNP mRNA was silenced" in "PNP expression was reduced through specific PNP siRNA transfection". It has to be further underlined that this experiment has been conducted to test the specificity of PNP antibody, so, even if the transfection efficacy is low, a partial reduction of PNP expression is enough to demonstrate the antibody specificity.

-*(p.19) 'Measurement of LDH activity performed on the same medium samples from all cultured cells showed no significant modification of the enzyme activity, with values ranging from about 2.2 ± 0.9% (1.5 h) to 4.2 ± 1.1% (24 h) of the total cellular LDH'. It is great that the authors assessed LDH outflow. They did so by quantifying a ratio of extracellular versus total activity of LDH. They reached a value of 2-4%. This should be compared with the percentage of PNP recovered in the media in relation to the total PNP activity, to gauge if the amount of extracellular PNP is 'several-fold' larger than that of LDH. Only this comparison will allow estimating the contribution of cell disruption for the outflow of PNP.*

R: The densitometric analysis of the blots in Fig. 2 shows a greater amount of PNP protein at 24 h than that revealed at 1.5 h in samples of extracellular medium from the different cell cultures under study. The lack of detectable immunobands of β-actin, a cell cytoskeleton protein, in the same samples is against a leakage of PNP from dying cells as the source of the enzyme found in the medium. This lack also forced us to include in the figures of this paper panels with Red Ponceau staining of blots to point out that equal amounts of samples were loaded for the electrophoretic run/blotting.
In addition, the activity of LDH, commonly assumed as an index of cell death, showed no significant modification so that the values of enzyme activity ranged from about 0.53±0.18 mU/ml at 1.5 h to 0.69±0.2 mU/ml at 6 h and 0.85± 0.22 mU/ml (the first and the last values corresponding to the percentage of 2.2 ± 0.9 and 4.2 ± 1.1 of the total cellular LDH, respectively, previously indicated in the text of the Results).
All these findings are in favor of a constitutive release of the enzyme from live cells.

-(p.20) 'microglia (0.891±0.09 nmoles/min/ml)>astrocytes (0.62±0.08 nmoles/min/ml)>>CGNs (0.41±0.03 nmoles/min/ml)'. I don't understand the basis to indicate that the difference between 0.89 and 0.62 (which is 0.27) is smaller than the difference between 0.62 and 0.41 (0.21), as suggested by the '>' and '>>'.
R: we corrected the sentence according to the reviewers' suggestion.

-(p.20) 'a transient PNP mRNA silencing'. Efficiency of transfection in the different cell cultures needs to be determined and communicated to evaluate the meaning of these determinations.
R: see the reply above reported for the same issue. To precisely describe the experiment, we changed the sentence reported by the referee with "we reduced PNP expression by using PNP specific siRNA (see methods for transfection efficacy)".

-(p.21) 'ATP is released from damaged cells and acts both as a cytotoxic factor and a pro-inflammatory mediator, being a universal "danger" signal (Durando et al., 1998)'. Consider revising the study quoted to illustrate this concept. The quoted studies should have its core dedicated to the concept to be illustrated, such as (di Virgilio REF; Rodrigues et al., Front Cell Neurosci 9:148).
R: as previously mentioned, we eliminated the cited reference, introducing the one suggested by the reviewer.

-(p.22 and throughout the text) 'remarkably'. Avoid using adjectives, especially the repetitive use of the same adjective.
R: we tried to eliminate repetitions of the same word.

-(p.22) 'in a dose-dependent manner'. No dose was used in this study, only defined concentrations of drugs were tested.
R: we agree and corrected the indicated word.

-(p.22) 'A438079 (10 μM) completely blocked the Bz-ATP effect'. This is pleonasm. A blockade is always a none versus all effect. If it is not complete it is not a blockade, but an inhibition or attenuation.
R: we corrected the sentence as suggested by the reviewer.

-(p.23) 'we silenced mRNA of the vesicular SNARE'. The efficiency of transfection was not reported to sustain this claim.
R: we added the requested information. As reported in the text of the manuscript, VAMP7 siRNA was performed following the manufacturer's protocol of the TriFECTa Dicer-Substrate RNAi kit (IDT) and the TransIT-TKO transfection reagent (Mirus). In preliminary experiments, TYE™ 563 DS fluorescent-labeled transfection control duplex was used, coupled to nuclear DAPI staining that allowed us to estimate the number of live cells. The efficiency of transfection by fluorescence microscopy was about 70% at 16 h after the induction. A similar result was obtained using a positive control (HPRT-S1 DS Positive Control duplex) for quantitative real-time RT-PCR.

-(p.23) 'Western blot analysis showed a remarkable reduction of VAMP7 protein'. Without a calibration, it is IMPOSSIBLE by Western blot analysis to estimate the extent of decrease of a protein: a reduction of 90% of immunodensity can correspond to a 5% change in the density of the protein (which may be 'remarkable' only without calibration).
R: From the efficiency of siRNA transfection for VAMP7 reported above, the decrease observed in the protein content by Western blot analysis was compatible with such a reduction and it was, therefore, remarkable.

Figures:
-Fig.1: The graph bars in panels A and B look so much alike that make me ask the authors if they may not have duplicated them.
R: We apologize for our mistake. We substituted the wrong panel with the right one.

-Fig.2: Without a lane of molecular weight markers, it is impossible to read the Ponceau staining to gauge the amount of proteins recovered in the analyzed media. It is also not possible to judge if the purported selective anti-PNP antibody only stains a simple band in the Western analysis.
R: As stated above, Ponceau staining was included only to demonstrate an equal loading of the samples loaded for the electrophoretic run (and their ~~the~~ good transfer after blotting). In fact, we did not have the possibility to use the current intracellular proteins to do this, as live cells do not normally release them outside. Moreover, we enclosed in the supplementary materials the image of a western blot analysis of conditioned media from neurons, astrocytes and microglia from 2 different experiments, showing that PNP antibody substantially identified only a major band with a molecular weight around 30-35 KDa (as reported for C6 glioma cells; Giuliani et al., 2016). This finding, together with the decrease in the PNP immune-band in

samples derived from microglia silenced for the enzyme expression demonstrates the specificity of the PNP antibody.

-Fig.6: What is the meaning of 'siRNA induction' in panel B? Is it a simple transfection of siRNA or is there some viral construct prone to induction?
R: As in the other experiments reported in the manuscript, it was a simple transfection of siRNA. So we corrected the indicated word.

Discussion:
-(p.25) 'LDH, index of cell necrosis'. Any dying cell will eventually release LDH.
R: We know that LDH may be released from dying cells but in general it is considered an index of necrosis whereas for apoptotic cells there are other more suitable markers such as staining of nuclei with DAPI or Hoechst, evaluation of activated caspases etc. Anyway, we substituted the word "necrosis" with the more generic expression "cell death".

-(p.26) 'leading also to activation of P2X7R in glial cells (Franke et al., 2006; Skaper et al., 2010; Weisman et al., 2012)'. This is somewhat abusive, in view of the fact that ATP can activate a myriad of P2 receptors and can be extracellularly converted to adenosine indirectly triggering the activation of adenosine receptors. Please consult a recent review on the INTEGRATED role of extracellular ATP in the brain (Rodrigues et al., 2015, Front Cell Neurosci 9:148).
R: The authors are aware that ATP or its metabolite adenosine may activate different receptors and, indeed, the sentence includes the word "also" just to indicate this aspect. The prevalence of the activity mediated by P2X7R in this paper is corroborated by the evidence that the antagonist of these receptors inhibited not only Bz-ATP- but also ATP-induced effect on PNP amount/activity. Moreover, in the discussion we made some considerations about the activity of suramin (even though data are not reported in the Results), that, unfortunately, was not suitable to resolve this aspect.

-(p.26) There is fundamental difference between ATP and LPS actions on glial cells: while both can trigger the release of pro-inflammatory signals, ATP is far more efficient than LPS to trigger the proliferation of glial cells. It is also 'remarkable' to remark that the amount of PNP in the medium of each cell type correlates with the proliferation rate of this cell type in culture.
R: We thank the reviewer for this suggestion that we included in the manuscript. Even though the period during which we evaluated the activity of LPS or ATP on PNP activity was very short (2 h), ATP might trigger glial proliferation (which, however, could be really evident only in the following 24-36 hours) eventually causing an increase in the extracellular PNP activity/amount.

-*(p.27) 'calcium release from the sarcoplasmic reticulum'. All eukaryotic cells have an endoplasmatic reticulum; the sarcoplasmatic reticulum is the particular name of this organelle in muscle cells.*
R: we corrected the sentence as requested, and also changed the cited reference, as the previous one was referred to the effect of suramin on ryanodine receptors in muscle cells endowed with sarcoplasmic reticulum.

-*There is considerable heterogeneity in the presentation of the references.*
R: we revised the references and corrected the errors.

HIGHLIGHTS

Purine Nucleoside Phosphorylase (PNP) is usually regarded as a cytosolic enzyme

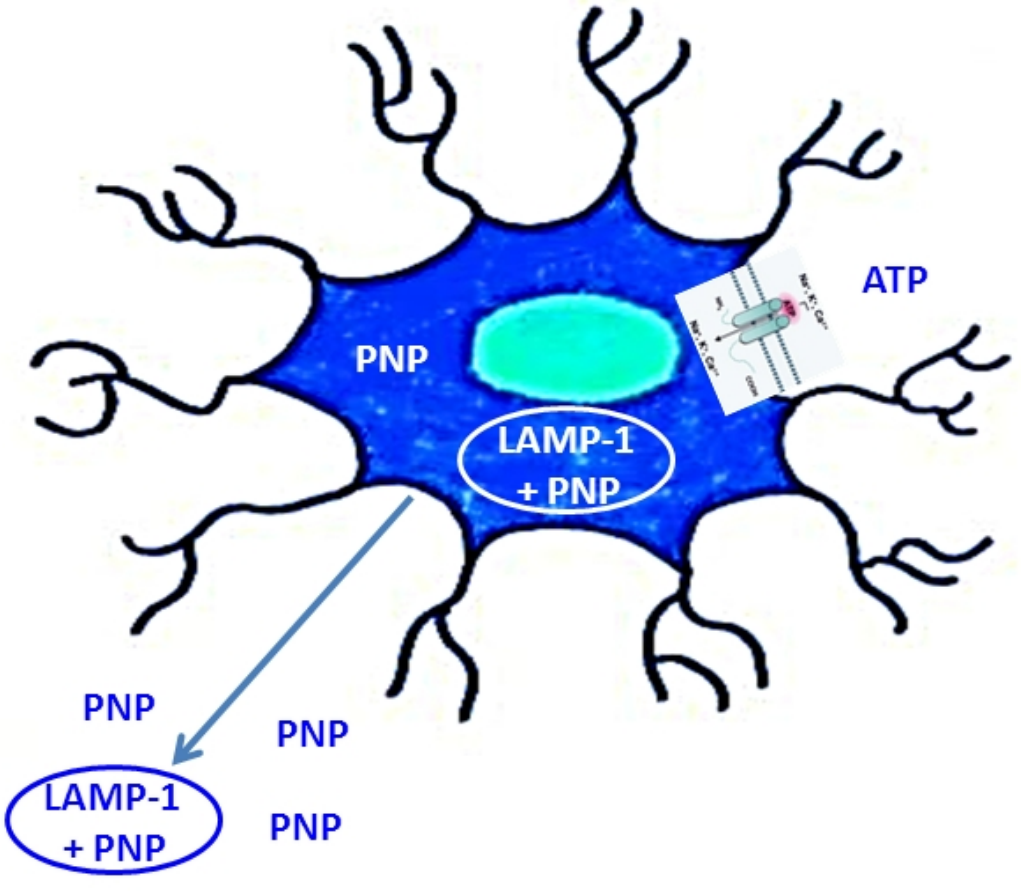
PNP is also constitutively released from cultured neurons and glial cells

PNP release from glial cells is stimulated by ATP but not by lipopolysaccharide

ATP-enhanced PNP release is mediated by $P2X_7$ receptors

ATP-induced PNP release partly occurs through secretion of lysosomal vesicles

PNP RELEASE FROM GLIAL CELLS



Neural cells, mainly astrocytes and microglia, constitutively release PNP in the pericellular fluid. Extracellular ATP interacting with P2X7 receptors increases such a release, in part involving the lysosomal secretory pathway.

RELEASE OF SOLUBLE AND VESICULAR PURINE NUCLEOSIDE PHOSPHORYLASE FROM RAT ASTROCYTES AND MICROGLIA INDUCED BY PRO-INFLAMMATORY STIMULATION WITH EXTRACELLULAR ATP VIA $P2X_7$ RECEPTORS

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Abbreviations: A-CM, astrocyte derived-conditioned medium; BME, Basal Medium Eagle; Bz-ATP, 3'-O-benzoyl-benzoyl-ATP; CGNs, cerebellar granule neurons; CNS, central nervous system; CSF, cerebrospinal fluid; JNK, c-Jun N-terminal kinase; DTT, dithiothreitol; ELISA, enzyme linked immunosorbent assay; EVs, extracellular vesicles; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GUA, guanine; GUO, guanosine; IL-1β, interleukin-1β; LAMP-1,

Lysosome-Associated Membrane Protein-1; LPS, lipopolysaccharide; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; M-CM, microglia derived-conditioned medium; N-CM, neuron derived-conditioned medium; PBS, phosphate buffered saline; PF, pellet fraction; PNP, purine nucleoside phosphorylase; $P2X_7R$, $P2X_7$ receptor; RT, room temperature; SDS, sodium dodecyl sulphate; SF, soluble fraction; VAMP7, Vesicle-associated membrane protein 7; XOR, xanthine oxidoreductase.

Abstract

Purine nucleoside phosphorylase (PNP), a crucial enzyme in purine metabolism which converts ribonucleosides into purine bases, has mainly been found inside glial cells. Since we recently demonstrated that PNP is released from rat C6 glioma cells, we then wondered whether this occurs in normal brain cells. Using rat primary cultures of microglia, astrocytes and cerebellar granule neurons, we found that in basal condition all these cells constitutively released a metabolically active PNP with Km values very similar to those measured in C6 glioma cells. However, the enzyme expression/release was greater in microglia or astrocytes that in neurons. Moreover, we exposed primary brain cell cultures to pro-inflammatory agents such as lipopolysaccharide (LPS) or ATP alone or in combination. LPS alone caused an increased interleukin-1β (IL-1β) secretion mainly from microglia and no modification in the PNP release, even from neurons in which it enhanced cell death. In contrast, ATP administered alone to glial cells at high micromolar concentrations significantly stimulated the release of PNP within 1 h, an effect not modified by LPS presence, whereas IL-1β secretion was stimulated by ATP only in cells primed for 2 h with LPS. In both cases ATP effect was mediated by $P2X_7$ receptor ($P2X_7R$), since it was mimicked by cell exposure to Bz-ATP, an agonist of $P2X_7R$, and blocked by cell pre-treatment with the $P2X_7R$ antagonist A438079. Interestingly, ATP-induced PNP release from glial cells partly occurred through the secretion of lysosomal vesicles in the extracellular medium. Thus, during inflammatory cerebral events PNP secretion promoted by extracellular ATP accumulation might concur to control extracellular purine signals. Further studies could elucidate whether, in these conditions, a consensual activity of enzymes downstream of PNP in the purine metabolic cascade avoids accumulation of extracellular purine bases that might concur to brain injury by unusual formation of reactive oxygen species.

Keywords: brain cell cultures; extracellular purine nucleoside phosphorylase (PNP); P2X7 receptor; mechanism of release; lysosomal vesicles.

Chemical Compounds:

ATP: CID 5957

LPS: CID 11970143

Bz-ATP: CID 115205

SB202190 : CID 5353940

SP600125: CID 8515

A438079: CID 11673921

1. Introduction

Purine Nucleoside Phosphorylase (PNP) is a ubiquitous enzyme that plays a crucial role in the metabolism of purines (Bzowska et al., 2000) by catalyzing the cleavage of ribonucleosides to generate purine bases. In humans, the PNP gene is located on chromosome 14 (14q13.1) and the PNP protein, a trimer of approximately 90,000 daltons, has been found in most body tissues, with the highest levels in lymphoid tissue. Accordingly, PNP inhibitors behave as T-cell selective immune suppressants and are used for treating many forms of leukemia (Balakrishnan et al., 2010; Bantia and Kilpatrick, 2004). On the other hand, there are conditions in which point mutations are responsible for PNP deficiency, leading to a loss of catalytic function (Williams et al., 1987; Aust et al., 1992; Pannicke et al., 1996; Dalal et al., 2001; Moallem et al., 2002). Deficiency of PNP causes a rare inherited disease determining severe combined immunodeficiency (Market, 1991), the most pathological features of which are recapitulated in a PNP knock-out mouse (Arpaia et al., 2000). As expected, PNP-deficient patients suffer from recurrent infections and autoimmune diseases, but they also present serious neurological disorders (Zabay et al., 1982). In relation to the nervous system, PNP histochemical localization seems to be restricted to glial cells, whereas its expression in neurons is still debated (Castellano et al., 1990; Zamzow et al., 2008). Dalmau and collaborators (1998) additionally showed a different PNP staining in microglial cells depending on microglial morphology and developmental stage of normal central nervous system (CNS). However, they observed PNP labeling also in blood vessels, some nerve fibers and astrocytes, suggesting a complex mechanism of cell interactions in the regulation of extracellular purine levels. Until recently, data on the presence of PNP in the extracellular compartment have been lacking, and PNP activity had only been measured in rat cerebrospinal fluid (CSF) (Silva et al., 2004). Yet, the metabolic activity of this enzyme outside the cells would be important by concurring to clarify the pericellular fluid from purine nucleosides, which behave as signal molecules interacting with specific membrane receptors. In this way, extracellular PNP would contribute to assure a coordinated control of the purinergic activity. With this in mind and on the

basis of the evidence that PNP levels are increased in tumors (Maeda et al., 1992; Kojima et al., 2012), we investigated whether PNP may be released from cancer cells such as those from rat C6 glioma cell lines, finding that this actually occurred (Giuliani et al., 2017). Then, we wondered whether PNP release is a physiological event occurring from "normal" (non tumoral) cells. To this aim, we used primary astrocyte and microglial cell cultures in comparison with neuronal cultures, choosing cultures of cerebellar granule neurons (CGNs), since the cerebellum is the prevailingly affected brain area in PNP deficiency (Mansouri et al., 2012). Moreover, we evaluated whether PNP release might be modulated by stimuli implicated in brain injury such as lipopolysaccharide (LPS) and ATP. Both agents are widely known, the former as a pro-inflammatory agent also at the CNS (Huang et al., 2008) and the latter as an ancestral and ubiquitous substance that is released virtually from all cells and achieves high extracellular concentrations during acute and inflammatory brain damages (Di Virgilio et al., 2009; Rodrigues et al., 2015) We found that PNP is released mainly from glial cells and that ATP (at high micromolar concentrations) but not LPS increased the enzyme release by activating $P2X_7$ receptors ($P2X_7R$). Additionally, our findings demonstrated that this ATP-induced effect partly occurred through a new, unconventional pathway represented by lysosomal extracellular vesicles (EVs), also implied in the secretion of inflammatory cytokines such as interleukin 1β (IL-1β) from immune effector cells upon $P2X_7R$ stimulation (Dubyak, 2012)

2. Materials and Methods

A total of 175 animals were used for all experiments. All animal experiments were authorized by the University of Bologna bioethical committee (Protocol n° 17-72-1212) and performed according to Italian and European Community laws on animal use for experimental purposes.

2.1. Preparation of brain cell cultures

Mixed glial cell cultures were prepared from cerebral cortex of newborn Wistar Han Outbred rat pups, as previously described (Levi et al. 1993). Briefly, after removal brain tissue was cleaned

from meninges, trypsinized for 15 min at 37°C and, after a further mechanical dissociation, the cell suspension was washed and plated on poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA, 10 μg/ml) 75 cm^2 coated flasks. Mixed glial cells were cultured for 10-13 days in Basal Medium Eagle (BME, Life Technologies Ltd, Paisley, UK) supplemented with 100 ml/L heat-inactivated fetal bovine serum (FBS, Life technologies), 2 mmol/L glutamine (Sigma-Aldrich) and 100 μmol/L gentamicin sulphate (Sigma-Aldrich).

Microglial cells were harvested from mixed glial cells cultures by mechanical shaking, resuspended in fresh medium without serum and plated on uncoated 35 mm dishes at a density of 1.5 x 10^6 cells/1.5 ml medium/well. Cells were allowed to adhere for 30 min and then washed to remove non-adhering cells. Then cultures were maintained in standard condition (37°C, 5% CO_2) and medium was replaced every three days.

For the preparation of astrocyte cultures, 10-day-old primary mixed glial cultures were vigorously shaken to detach microglia and oligodendrocytes growing on top of the astrocytic layer. The remaining adherent cells were detached with trypsin (0.25%)/EDTA (Life Technologies), and the resulting cell suspension was left at room temperature (RT) in uncoated flasks to allow adherence of microglia to the plastic surface. After 20–30 min, non-adherent or loosely adherent cells were collected after mild shaking of the flasks, and the adhesion step was performed once more. Supernatants containing non-adherent cells were collected and centrifuged; cells were resuspended in fresh BME medium without serum (Life Technologies) and reseeded on poly-L-lysine-coated (Sigma-Aldrich) 35 mm Ø dishes at a density of 1.5 x 10^6 cells/1.5 ml medium/well. Afterwards, cells were cultured until their use. Flow cytometric analysis showed that the cultures obtained by this way were enriched astrocytes, with about 95% of the cells staining for the glial marker, glial fibrillary acidic protein (GFAP), and less than 2% staining for the microglial marker, cd11b/c (Pharmingen BD Bioscience, San Diego, CA, USA), as previously indicated (Di Iorio et al., 2002).

Finally, primary cultures of CGNs were prepared from 7 day-old Wistar strain rats, as previously described (Gallo et al., 1987). Animals were rapidly anesthetized with an ice-cold treatment and cells were dissociated from cerebella, plated on 35 Ø mm dishes or in 24 well plates, previously coated with 10 μg/ml poly-L-lysine, at a density of 2 x 10^5 cells/cm^2 in BME supplemented with 100 ml/L heat-inactivated FBS (Life technologies), 2 mmol/l glutamine, 100 μmol/l gentamycin sulphate and 25 mmol/l KCl (all from Sigma-Aldrich). Sixteen hours later, 10 μM cytosine arabinofuranoside (Sigma-Aldrich) was added to avoid glial proliferation. After 7 days *in vitro*, differentiated neurons were shifted to serum-free BME medium containing 25 mmol/l KCl and used for the experiments.

2.2. Cell culture treatments

For detection of PNP protein release from rat microglia, astrocytes and cerebellar neurons, cell cultures were grown for different periods (1.5, 6 or 24 h) in BME medium without serum whereas for measuring IL-1β and lactate dehydrogenase (LDH) release cells were stimulated for 24 h with ultrapure LPS (0.1, 0.5 or 1 μg/ml; Invivogen, San Diego, CA, USA), agonist for TRL4 receptors. In some experiments, antagonists of some members of the mitogen activated protein kinase (MAPK) such as SB202190 (10μM, Sigma-Aldrich) or SP600125 (10 μM, Sigma-Aldrich) were added to the culture medium 1 h before the exposure to LPS (1 μg/ml) for 24 h. In another set of experiments, we evaluate the ability of ATP (Sigma-Aldrich) at high concentrations (500 or 1000 μM) or 3'-O-benzoyl-benzoyl-ATP (Bz-ATP, 125 or 250 μM) (Tocris, Space Import Export, Milan, Italy), to affect the release of the pro-inflammatory cytokine IL-1β or PNP from astrocyte and microglial cell cultures. As previously reported (Perregaux and Gabel, 994), in order to obtain an appreciable IL-1β secretion from glial cells a cell pre-treatment for 2 h with 1 μg/ml LPS (known as cell priming) was performed prior to ATP or BzATP treatment. When present, the competitive $P2X_7$ receptor antagonist A438079 (10 μM) (from Tocris), was added to the culture medium 1 h prior ATP or BzATP treatment.

2.3. Immunocytochemistry

Microglial, astrocyte and differentiated cerebellar neuron cell cultures were fixed for 15 min in 4% paraformaldehyde at RT. Non-specific sites were blocked with 0.1% normal goat serum and 3% bovine serum albumin (both from Sigma-Aldrich) for 1h at RT. After several washes, cells were incubated overnight at 4°C with a rabbit anti-rat PNP antibody (rat VSILMESIPPRERAN C-term antigen, IgG fraction of antiserum; Neosystem, Strasbourg, France; 1:2000 dilution) alone or together with a mouse anti-Lysosome-Associated Membrane Protein-1 (LAMP-1, rat 4E151 epitope, Santa Cruz Biotechnology, Santa Cruz, CA, USA, cat. no. sc-71489, 1:500 dilution) antibody and further incubated with secondary antibodies for 1.5 h at RT (goat anti-rabbit FITC cat no. F0382, alone or together with goat anti-mouse TRITC cat no. T5393, both from Sigma-Aldrich). Lastly, nuclei were stained with Hoechst 33258 (0.1 mg/ml, Sigma-Aldrich, cat no. B1155) for 5 min at RT. Stained cultures were photographed with a fluorescence microscope (Eclipse TE 2000-S microscope, Nikon, Tokyo, Japan) equipped with an AxioCam MRm (Zeiss, Oberkochen, Germany) digital camera by use of a x 60 objective.

2.4. Real time PCR

In order to detect PNP mRNA expression, real time PCR was performed on total RNA extracted from microglia, astrocytes and cerebellar granule neurons. To this aim 3×10^6 cells were directly lysed in 1 ml of Tri-reagent (Sigma-Aldrich), according to the manufacturer's protocol. RNA pellets were resuspended in diethyl pyrocarbonate (DEPC; Sigma-Aldrich)-treated deionized water. RNA levels were quantified using a NanoDrop UV spectrophotometer (Thermo Scientific, NanoDrop Products, Wilmington, DE, USA) and stored at −80°C until used for cDNA synthesis. For each sample, 1 μg of DNase-treated RNA (Thermo Fisher Scientific, Waltham, MA, USA cat. no. EN0525) was retro-transcribed using the Superscript III First-Strand Synthesis SuperMix for

qRT-PCR kit (Life Technologies) following the manufacturer's protocol. cDNAs were stored at −20°C until used for qRT-PCR.

Rat PNP (accession number NM_001106031.1) and β-actin (accession number NM_031144) qRT-PCR primers were designed using the software Beacon Designer 7.2 (PREMIER Biosoft International, Palo Alto, CA, USA). The following primer pairs (from Sigma Genosys) were used:

Rat PNP

Forward: 5' ATCCGTGACCACATCAACCT 3' Tm = 55.9 °C

Reverse: 5' TTGAAAGCCTTCTGCCTCAT 3' Tm = 54.3°C

Product size: 135 bp

Rat β-Actin

Forward: 5′ AGCAGATGTGGATCAGCAAG 3′ Tm = 54.9°C

Reverse: 5′ AACAGTCCGCCTAGAAGCAT 3′ Tm = 56.0°C

Product size: 81 bp

cDNA samples were diluted to a concentration of 20 ng/μl. A master mix of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Segrate, Italy) was mixed with the adequate primers (final concentration 0.2 μmol/l), cDNA (40 ng per replicate) and deionized water. qRT-PCR was performed using an iCycler IQ (Bio-Rad) thermal cycler. Samples were tested in triplicate.

Relative gene expression was calculated using the $\Delta\Delta^{Ct}$ method, where Ct represents the cycle threshold. Δ^{Ct} values were calculated as difference between the target gene and the expression of the endogenous gene β-actin and $\Delta\Delta^{Ct}$ values were expressed as percentage of values measured for CGNs.

2.5. Western blot analysis

To analyze the intracellular or extracellular PNP protein expression in microglial, astrocytes and cerebellar neuron cultures in the absence or in the presence of the above-mentioned pharmacological treatments, cells were directly collected in loading buffer 2X (0.5 mol/l Tris-HCl

pH. 6.8; 40 gr/l sodium dodecyl sulphate (SDS); 20 ml/l glycerol; 2 gr/l bromophenol blue; 0.2 mol/l dithiothreitol (DTT); all chemicals were from Sigma-Aldrich), while inorganic salts were removed from media, which were further concentrated by using AmiconYM-3 (EMD Millipore Corporation, Billerica, MA, USA). In detail, 1.5 x 10^6 cells from each 40mm Ø microglia or astrocytes well and 2.4 x 10^6 cells from each 40mm Ø cerebellar neurons well were collected in 50 µl of ice-cold lysis buffer (50 mM Tris/HCl; 1 mM EDTA; 1% SDS; protease inhibitor cocktail; all chemicals were from Sigma-Aldrich) and total protein content was measured by the Lowry method (Lowry et al., 1951). In parallel, from 1 ml aliquots of the corresponding conditioned media from microglia, astrocytes or CGNs (respectively named M-CM, A-CM and N-CM) for each condition, 500 µl were lyophilized by using Microcon-YM-3 (Millipore), and resuspended in 15 µl of Loading buffer 2X. Fifteen µl/lane of concentrated media or 20 µg of total cell homogenates were loaded onto a 15% (wt/vol) SDS-polyacrilamide gel (Bio-Rad). After electrophoresis and transfer onto nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, UK), membranes were stained in some cases with Ponceau Red S 0.1% in acetic acid 5% for 5 min at RT as loading and transfer control (Romero-Calvo et al., 2010), and further washed in bis-distilled H_2O. Membranes were then blocked for 1 h with a Blocking Solution made of 50 gr/l non-fat dried milk (Bio-Rad)/1 ml/l Tween 20 in phosphate buffer solution (Sigma-Aldrich), pH 7.4, and incubated overnight at 4°C with a custom rabbit anti-PNP primary antibody (rat VSILMESIPPRERAN C-term antigen, IgG fraction of antiserum; Neosystem, Strasbourg, France; 1:2000 dilution) or, as for the experiments of Vesicle-associated membrane protein 7 (VAMP7) silencing, with a rabbit polyclonal anti-VAMP7 antibody (18 aminoacid sequence near the N-term of human VAMP7, Origene, Rockville, MD, USA; cat. no. TA319712; 1:1000 dilution) in 1 ml/l Tween 20/phosphate buffered saline (PBS). In a set of experiments, membranes previously probed with anti-PNP primary antibody were re-probed with a mouse antibody against LAMP-1, the same used for immunocytochemistry (Santa Cruz Biotechnlogies). Subsequently, nitrocellulose membranes were incubated with a goat anti-rabbit secondary antibody, conjugated to horseradish peroxidase (Santa Cruz Biotechnology, cat. No. sc-

2004, 1:2000 dilution) for 90 min at RT in 1 ml/l Tween 20/PBS. When indicated, membranes were re-probed with a primary antibody against β actin (synthetic peptide (20-33) N-terminal epitope; Sigma Aldrich; cat. no. A5060; 1:100 dilution) to assure equal sample loading. Labeled bands were visualized by the enhanced chemiluminescence method (ECL, GE Healthcare) and quantified by densitometric analysis (Molecular Analyst System).

2.6. Measurement of PNP activity

The activity of PNP released in the culture medium was evaluated according to a recently developed simple and highly sensitive HPLC method based on the use of a fluorescence detector to exploit the natural fluorescence of guanosine (GUO) and guanine (GUA) that represent the substrate and the product of PNP reaction, respectively (Giuliani et al., 2016). Thus, $3x10^5$ astrocytes or $7.5x10^5$ microglia cells or $1.0x10^6$ cerebellar neurons were seeded on 35 mm Petri dishes and incubated for 6 h in Krebs-HEPES buffer, a simplified medium constituted by 15 mM HEPES, pH 7.4, 120 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, and 10 mM D-glucose (all from Sigma Aldrich). This medium was then removed and placed in suitable devices (Amikon Ultra 2 ml, cutoff 10 K; Merck Millipore, Darmstadt, Germany) by which it was possible to concentrate the PNP present in a volume of about 40 µl. These samples were stored at –80 °C until assayed. The enzymatic reaction mixture was composed of 50 mM HEPES pH 7.0, with or without 50 mM inorganic phosphate (Pi; used as co-substrate) plus a fixed aliquot of concentrated medium (as a source of PNP). The enzyme reaction started by the addition of varying concentrations of GUO (from 0 up to 200 µM). The mixture was incubated by shaking at 37◦C for 15 min. The reaction was stopped by heating the mixture at 70 °C for 5 min and precipitated proteins were removed by centrifugation at 10,000 x g for 10 min at 4 °C. The supernatant was filtered with 0.2 µm filters (Millipore, Vimodrome, Italy) before injection into the HPLC system. Reaction mixtures to which only substrate or medium were added, were run and served as blanks. Each sample was run in duplicate in at least three independent experiments.

The HPLC system (Agilent 1100 series; Waldbronn, Germany) was equipped with a thermostated column compartment, a diode array detector and a fluorescence detector (Agilent Technologies). Briefly, separation was obtained on a Phenomenex Kinetex pentafluorophenyl analytical column (5µm pore size, 100 Å particle size, 250×4.6 mm; Phenomenex INC, Castelmaggiore, Bologna, Italy) at 35◦C, using a mobile phase composed of 0.1% (v/v) formic acid in water (solution A) and methanol (solution B). Separation was carried out with a 15 min non-linear gradient elution while the flow rate (of the mobile phase) was set at 1ml/min. Autofluorescent GUO and GUA were monitored at an excitation wavelength of 260 nm and an emission wavelength of 375 nm while eventual other purine compounds present in the eluate were spectrophotometrically monitored at 254nm. All substances were identified and quantified by comparison with pure external standards. PNP activity was expressed as nmol of GUA formed per min per ml.

2.7. Lactate dehydrogenase assay

LDH activity was determined by a commercially available kit (Promega Italia, Milan, Italy). Cells were seeded ($3x10^3$ cells/well) in 96-well plates and grown for 4 days. Then, after culture medium replacement with Krebs-HEPES buffer, cells were incubated in standard condition in the presence or not of LPS. In some experiments cells were incubated with inhibitors (SB202190 or SP600125) of some mitogen-activated protein kinase (MAPK) 1 h prior to LPS addition. Afterwards, media were collected and stored whereas cells in the plate were incubated at 37 °C and 5% CO_2 for 45 min with a specific lysis solution and then centrifuged at 250 g for 4 min. Subsequently, 50 µl aliquots from all collected media and wells were transferred to a new 96-well plate, and 50 µl of substrate buffer consisting of 0.7 mM p-iodonitrotetrazolium Violet, 50 mM L-lactic acid, 0.3 mM phenazine methoxysulfate, 0.4 mM NAD and 0.2 M Tris-HCl pH 8.0 were added. The plate suitably blanket was protected from light and incubated at RT for 30 min. Reaction was blocked by addition of 50 µl/well of stop solution. The absorbance was spectrophotometrically measured at 490 nm and the results were expressed either as milli-Unit/ml measured in the extracellular culture medium at

different times or as a percentage of total LDH released from the positive control consisting of cells treated with lysis solution and calculated as follows: (medium absorbance value - white absorbance value)/(medium absorbance + lysate absorbance) x 100.

2.8. Enzyme linked immunosorbent assay (ELISA) for IL-1β evaluation

Cultured cells were plated in 24-well tissue culture plates at $1x10^5$ cells/well and stimulated for 24 h with LPS (10 µg/ml) in the presence or absence of an inhibitor of p38 MAPK (SB2012190) or JNK (SP600125). Cell-free supernatants were collected and stored at -80°C until analysis. IL-1β levels in the supernatants were examined by ELISA kit (R&D Systems) with detectable limits of 21–4000 pg/ml, in accordance to the manufacturer's protocol. Cell lysates were also prepared and the Bio-Rad protein assay was performed to measure total cellular protein from each of the cell groups. Results are shown as mean picograms (pg) of cytokine per milligram (mg) of total cell protein.

2.9. PNP and VAMP7 silencing

For PNP small interfering RNA (siRNA) technique, microglial cultures were prepared as above described. Using these cells, selective glial PNP silencing was obtained through transfection by using LipofectAMINE2000 Reagent with commercially available siRNA against rat PNP (previously tested for efficacy by the Rosetta bioinformatics system) or with a universal siRNA negative control (Sigma-Aldrich). The glial specificity of siRNA transfection was due to the fact that mature CGNs were not transfected by LipofectAMINE2000 in our hands (data not shown). Cultures were transfected in OptiMEM/serum-free BME (all from Life Technologies Italia) without antibiotics, following the manufacturer's protocol. After 2 h, the medium with the LipofectAMINE-DNA complex was replaced with serum-free BME. Twenty-four hours after siRNA transfection, cells were collected for Western blot analysis. The vector pmaxGFP® (Amaxa, Cologne, Germany) was used to test transfection efficiency in both microglia and CGNs primary cultures, as previously described (Peña-Altamira et al., 2014; Polazzi et al., 2013), showing that when using

LIPOFECTAMINE-2000, while GFP-positive neurons were almost undetectable in CGN cultures, microglial GFP-positive cells were about 17±2% respect to total Hoechst stained cells.

For siRNA-mediated knockdown of VAMP7, astrocytes or microglia grown as previously described were plated onto 6 well flat bottom multiwell plate (Falcon®) in DMEM (Dulbecco's modified Eagle's medium; Gibco®) supplemented with 5% FBS without antibiotics, at 37°C in the presence of 5% CO_2. Cells were seeded at sub-confluence and transfected on the next day with TriFECTa Dicer-Substrate RNAi kit (Integrated DNA Technologies) specific to VAMP7, at 25 nM concentration per well using transIT-TKO transfection reagent (Mirus BioCorporation, Madison, WI, USA). Transfection was performed as described by manufacturer's protocol. The TriFECTa kit also provided control sequences for siRNA experiments, including an HPR T-S1 positive transfection control RNA duplex, a TYE™ 563 DS fluorescent-labeled transfection control duplex and a scrambled universal negative control RNA duplex, the last one absent in human, mouse and rat genomes. Preliminary experiments were carried out either by fluorescence microscopy using TYE™ 563 DS coupled to nuclear DAPI staining to estimate the number of live cells or by quantitative real-time RT-PCR using the positive control HPRT-S1 DS and the scrambled negative control. In both cases, the efficiency of siRNA transfection was maximal and of about 70% at 16 h from the beginning of the experiment.

2.10. Preparation of extracellular vesicles from conditioned medium

Fresh, 2h serum-free medium was collected from primary cultures of either microglia or astrocytes, treated or not with 500 µM ATP, centrifuged for 10 min at 10.000 x g, at RT, filtered through 0.22 µm filters and immediately ultracentrifuged for 2 h at 25.000 x g, 4°C. Aliquots of CM or of supernatant (SF, 500 µl) obtained by medium ultra-centrifugation were concentrated by using Microcon-YM-3 (Millipore) and then resuspended in 20 µl 2X Loading Buffer, while pellets (PF) were collected in 20 µl 2X LB for Western blot analysis of PNP and LAMP-1 expression.

2.11. Statistical analysis

All quantitative data are presented as means ± S.E. from independent experiments. Statistical significance between different treatments was calculated with GRAPHPAD PRISM 6 (La Jolla, California, USA) by using two-tailed Student's t-test or one-way analysis of variance (ANOVA) followed by post-hoc comparison through Dunnett's test. A value of $p<0.05$ was considered statistically significant.

3. RESULTS

3.1. PNP expression in rat primary cultures of microglia, astrocytes and cerebellar granule neurons

First, we evaluated the intracellular PNP expression by different methods in rat primary microglia, astrocyte and CGN cultures. By real-time PCR the highest levels of PNP mRNA were found in microglial cells and the lowest ones in differentiated CGNs (Fig. 1A). Western blot analysis (Fig. 1B) confirmed the presence of PNP protein in all brain cells, with a more significant expression in glial cells than in CGNs. The specificity of the primary antibody against rat PNP, used also for the subsequent immune-fluorescence evaluation, was demonstrated by the significant decrease in the PNP electrophoretic band in samples derived from microglial cultures, in which PNP expression was reduced through specific PNP siRNA transfection, in comparison with wild type cells considered as control (Fig. 1D). The immune-fluorescent technique revealed a diffuse PNP staining in the nucleus and cytoplasm of microglia and astrocytes, while differentiated CGNs showed a prevailing nuclear labeling (Fig. 1C).

3.2. PNP release from cultured microglia, astrocytes and differentiated CGNs

We then investigated whether the PNP protein was secreted by cells. Western blot analysis carried out on the growth medium removed after different times from its renewal to the cultures demonstrated that PNP was constitutively released from microglia, astrocytes and to a lesser extent

also from CGNs (Fig. 2A). The electrophoretic band at 32 KDa was substantially the only one identified by the antibody used in this study (see Supplemental Material, Fig. 1S) and the molecular weight corresponds to that found in the literature and also in rat C6 glioma cells. Noteworthy, PNP tended to accumulate over a period of 24 h as demonstrated by the western blot analysis (Fig. 2B). In contrast, LDH activity assayed on the same medium samples from all cultured cells showed no significant modification of the enzyme activity, with values ranging from 0.53 ± 0.18 mU/ml at 1.5 h to 0.85± 0.22 mU/ml at 24 h. Thus, no significant increase in cell death occurred during the examined periods of time. The substantial absence of lytic processes in our cultures over a period of 24 h was also confirmed by the absence of cytosolic proteins such as β-actin in the medium assayed again by western blot analysis (data not shown). For this reason, in order to show that the electrophoretic samples were loaded to a similar extent, we were forced to stain our blots by Red Ponceau S (see Fig. 2 and the other ones).

Subsequently, we evaluated whether secreted PNP was metabolically active. To this aim, cells were incubated for 6 h in a simplified medium and aliquots from all brain cultures were analyzed in order to measure the ability of the enzyme to convert the substrate GUO into the corresponding product, GUA (Fig. 2B). Within the range of GUO concentrations used to reach saturation (from 0 up to 200 µM), the curves were best fitted by non-linear regression analysis to the Michaelis-Menten hyperbolic function (Fig. 2b, curves with filled circles). The Km values were of about 24 µM, without significant differences among the cell types under study. In contrast, the maximal activity of the enzyme (Vmax) was greater for medium samples deriving from glial cells (microglia: 0.891±0.09 nmoles/min/ml; astrocytes: 0.62±0.08 nmoles/min/ml) than from CGNs (0.41±0.03 nmoles/min/ml). These findings were compatible with the data from western blot analysis above reported. To confirm that the obtained kinetic parameters were related to the effective enzyme activity, we reduced PNP expression by using PNP specific siRNA (see methods for transfection efficacy) or removed the essential co-substrate Pi from the reaction mixture. Considering that siRNA was effective only in glial cells, the former experimental condition significantly decreased

PNP activity in these cells whereas the latter almost completely abolished the ability of PNP to form GUA from the substrate GUO (Fig. 2B, curves with open squares or circles, respectively) in all cell types.

3.3. Cell exposure to LPS, a typical pro-inflammatory stimulus, did not modify the constitutive PNP secretion from cultured microglia, astrocytes and differentiated CGNs

Once assessed that PNP was actually released from cells under study, we wondered whether inflammatory agents, able to trigger acute brain diseases (infection, ischemia, trauma) could influence the constitutive PNP release from nervous cells. We first examined the influence of LPS, a well-known pro-inflammatory stimulus, exposing our cultures for 24 h to increasing concentrations of ultrapure LPS (0.1; 0.5 and 1 μg/ml). In the CM collected at the end of this period and analyzed by western blotting none of the assayed LPS concentrations affected the amount of released PNP from microglia, astrocyte or CGN cultures (Fig. 3A-C). A very similar behavior was observed exposing our cultures to different dosages (1; 10; 100 ng/ml) of another pro-inflammatory agent such as interferon γ (data not shown). On the other hand, LPS alone at the highest dose (1 μg/ml) caused a modest although significant release of IL-1β from astrocytes, that could be referred to the amount of microglia contaminating astrocyte cultures (Facci et al., 2014), and to a greater extent from microglial cells, but not from or neurons. This stimulatory LPS effect was counteracted by pretreatment with antagonists of some members of the MAPK family such as SB202190 (10μM), a selective inhibitor of the p38 MAPK pathway, and SP600125 (10μM), able to antagonize different subtypes in the c-Jun N-terminal kinase (JNK) pathway. Both these pathways are involved in the production of pro-IL1β promoted by LPS (Facci et al., 2014). Noteworthy, the exposure of CGNs to LPS (1 μg/ml) for 24 h increased the LDH activity, an index of cell necrosis, in the culture medium whereas the same treatment did not modify LDH release from glial cultures. These results confirmed that LPS acted as a pro-inflammatory agent, being able to stimulate the release of IL-1β, a typical inflammatory cytokine, mainly from microglial cells through the activation of known

molecular pathways without affecting cells vitality. In contrast, LPS did not modify the PNP release from the same cells, even from neurons of which it caused an enhanced death.

3.4. ATP enhanced the PNP secretion from cultured microglia and astrocytes by acting through $P2X_7R$.

It is known that ATP is abundantly released from all brain cells, representing one of the most important neuro/glio-transmitters. Additionally, during pathological conditions, ATP is released from damaged cells and acts both as a cytotoxic factor and a pro-inflammatory mediator, being a universal "danger" signal (Di Virgilio et al., 2009; Rodrigues et al., 2015). Thus, we wondered whether ATP could affect/modulate PNP secretion from the cells under study, at the same time evaluating whether it stimulated IL-1β release from the same ones. Since we had previously observed that CGNs constitutively released very small amount of PNP whereas pivotal experiments demonstrated that ATP, like LPS, did not alter PNP and IL-1β secretion from CGNs at any assayed dose (data not shown), we focused on glial cells.

High concentrations (500 and 1000 μM) of ATP enhanced the PNP release in the extracellular medium from both cultured astrocytes and microglia, already after 1h (Fig 4A-B). In a parallel set of experiments we found that ATP (1000 μM) also induced the secretion of the pro-inflammatory cytokine IL-1β, but only when glial cells were exposed for 2 h to LPS (1 μg/ml) prior to ATP stimulation (Fig. 4C-D). Noteworthy, this LPS priming did not alter PNP release induced by the nucleotide from the same cells (data not shown).

Since ATP at high micromolar concentrations can activate $P2X_7R$, which are present in all brain cells (Franke et al., 2012; Sperlagh et al., 2006; Vekhratsky and Burnstock, 2014), we pre-treated both microglia and astrocyte cultures with A438079 (10 μM), a competitive antagonist of $P2X_7R$ (Bartlett et al., 2014). In this condition, the ATP-induced PNP release was decreased (Fig. 4A-B). As well, A438079 also reduced the IL-1β release induced by ATP in LPS-primed microglia (Fig. 4C-D).

To further support the role of $P2X_7$ receptor activation, we treated glial cell cultures with Bz-ATP, having care to use concentrations sufficiently selective to activate rat $P2X_7R$ (Young et al., 2007; Bartlett et al., 2014;). Western blot analysis of CM removed from microglia or astrocytes (Fig. 5A) showed that Bz-ATP (125 and 250 μM) stimulated PNP release from both types of cells in a concentration-dependent manner. Cell pre-treatment with the $P2X_7R$ antagonist A438079 (10 μM) blocked the Bz-ATP effect on PNP secretion (Fig. 5A). These findings were in favor of a role played by $P2X_7R$ in the ATP stimulation of PNP release. Also in this case, in a parallel set of experiments, we evaluated the ability of Bz-ATP (125 μM) to induce the secretion of the pro-inflammatory cytokine IL-1β. Like ATP, Bz-ATP enhanced the IL-1β release from microglial cultures previously exposed for 2 h to LPS (Fig. 5B) and the cell pretreatment with A438079 abolished the Bz-ATP effect on IL-1β release in both kinds of cells (Fig. 5B).

3.5. PNP release is mediated via a lysosomal secretory pathway

Since ATP is known to induce pro-inflammatory protein release through the lysosomal secretory pathway (Dubyak, 2012; Takenouchi et al., 2009a), we verified whether this modality was involved also in PNP release. Thus, we silenced mRNA of the vesicular SNARE tetanus neurotoxin-insensitive vesicle-associated membrane protein (TI-VAMP), also called VAMP7, a marker of secretory lysosomes (Takenouchi et al., 2009b; Rao et al., 2004) in both astrocytes and microglial cells. Western blot analysis showed a reduction of VAMP7 protein after 16 h from its silencing whereas in the following 24 and 48 h the protein expression returned to control values (Fig. 6A). Accordingly, the activity of PNP was significantly reduced in astrocyte and microglial CM collected at 16 h from VAMP7 silenced cells in comparison either to control cells or to ATP-treated cells (Fig. 6B).

Since glial secretion through extracellular vesicles (EVs) is becoming more and more important in physiological glial-neuron communication, we assessed whether PNP protein could be released from glial cells by this way. EVs were isolated from M-CM and A-CM removed after 2 h from

cultured cells maintained in basal conditions or exposed to 500 μM ATP. Western blot analysis revealed the presence of PNP in the soluble (SF) and pellet fraction (PF, containing EV) derived from M-CM and A-CM, mainly in medium removed from ATP-stimulated cells (Fig. 6C), being the pellet fraction also positive for LAMP-1. These data demonstrated that microglia and astrocytes released PNP also through both an EVs-mediated release, occurring *via* the lysosomal secretory pathway upon exposure to a high concentration of ATP. This was especially evident and statistically significant in microglial cells when comparing the pellet fraction from control and ATP treated cells.

4. DISCUSSION

The study of the PNP expression in the brain started in the early '90s, when it was demonstrated that in cerebral slices, PNP co-localized with GFAP, an astrocytic marker, and with OX-42, a microglial marker, suggesting that PNP is primarily expressed by glial cells and not by neurons (Castellano et al., 1990). Subsequently, Dalmau and collaborators (1998) reported PNP expression not only in microglial cells and astrocytes but also in blood vessels and in some nerve fibers, suggesting however, that glial cells are crucial for maintaining cerebral extracellular purine levels. In fact, differently from other tissues (Müller et al., 1983), *de novo* purine biosynthesis is reduced to low levels in the brain, and an important mechanism for producing purines by cerebral cells is the salvage pathway, in which extracellular purines and PNP are implicated. Thus, we undertook the present study with the aim to demonstrate not only the PNP expression in different brain cells but also to evaluate if these cells release the enzyme in the extracellular space. This study, indeed, was also favored by our previous experience with rat tumor cells, which proved to be able to release PNP that was also modulated by activation of some metabotropic purine receptors (Giuliani et al., 2017).

Here, using rat primary *in vitro* neuron, astrocyte and microglia cultures, we confirmed that PNP is expressed by glial cells, both at mRNA and protein levels, to a greater extent compared to neurons.

Additionally, we demonstrated that glial cells and also CGNs, although to the lowest extent, constitutively release PNP and that this extracellular PNP is active, being able to transform GUO, one of the possible substrates (the other ones are inosine and the deoxy-ribose GUO and inosine), into the corresponding metabolite, GUA. Noteworthy, PNP release was not linked to cell damages since the levels of LDH, index of cell death, were not changed in the extracellular medium over a period of 24 h during which those of PNP increased. It is also worth noting that the kinetic parameters were roughly in the same range of values reported in literature by other authors using different methods (Ghanem et al., 2008; Shewach et al., 1986) and the Km values were very similar to those determined in rat C6 glioma cells (Giuliani et al., 2017). Until now, no data have clearly demonstrated that PNP could be secreted by normal brain cells. Since PNP activity has previously been reported in CSF (Silva et al., 2004), we suggest that PNP in the CSF could derive from the physiological release from brain cells. Obviously, the presence of PNP in basal conditions suggests that it is involved in physiological functions of the CNS. It is well known that glial cells cooperate with neurons in fundamental processes, such as neural plasticity and neurogenesis as well as learning and memory, mainly through the release of soluble molecules (Bitzer-Quintero et al., 212; Yirmiya and Goshen, 2011), including purines. Also PNP has been implicated in learning and memory, since the exonic G/A single nucleotide polymorphism (SNP) in the PNP codifying gene, resulting in the amino acid substitution serine to glycine at position 51 (G51S), has been associated with a faster rate of cognitive decline in AD patients (Tumini et al., 2007). Moreover, exogenous GUO administered intraperitoneally to rats is rapidly metabolized by PNP to its metabolite GUA (Giuliani et al., 2012a), which has been shown to prevent amnesic effects caused by an inhibitor of nitric oxide synthase (NOS) activity (Giuliani et al., 2012b). Therefore, glial PNP release might be might be involved in the modulation of physiological processes related to learning and memory.

Additionally, we considered that microglia and astrocytes exert an immune function (Aguzzi et al., 2013) mainly in inflammatory processes following acute and chronic neurodegenerative diseases (Skaper et al., 2012; Collins et al., 2012; Eikelenboom et al., 2012), among which psychiatric

disorders are included (Shie et al., 2011). In these pathological conditions, glial cells become activated (Zielasek and Hartung, 1996) and release several factors. For this reason, we decided to study whether PNP release from brain cells could be modified by glial cell activation with neuro-inflammatory stimuli. We used purified LPS, the activity of which should occur by activating some members in the family of MAPK downstream LPS binding to the Toll-like receptor 4 (TLR4), as previously reported (Facci et al., 2014). Through this activity LPS is able to induce a wide range of inflammatory activities, *e.g.* increased phagocytosis, chemotaxis, cytokine secretion, activation of the respiratory burst and induction of NOS (John et al., 2003; Pawate et al., 2004). Additionally, we assayed the activity of ATP, which is released into the extracellular space either by exocytosis or from damaged and dying cells (Volonté et al., 2003), leading also to activation of $P2X_7R$ in glial cells (Franke et al., 2006; Skaper et al., 2010; Weisman et al., 2012). This could be reasonably considered as one of the first signals used by neurons to activate glial cells in order to increase their neuroprotective potential, *i.e.* by stimulating the release of neuroprotective molecules from glial cells (Skaper et al., 2010, 2011). Our results indicated that LPS, like interferon γ (data not shown), did not modify PNP release from any brain cell type, even from neurons in which it caused a certain cytotoxic/lytic effect. However, as an agent involved in triggering inflammatory brain conditions (Pawate et al., 2004), LPS was able to activate glial cells, but not neurons. In the medium from astrocytes it caused a modest increase in IL-1β release, likely due to microglial contamination of these cultures around 2-3%, whereas cytokine release from microglia was greater after 24 h. This effect could prevailingly be due to the release of the inactive cytokine pro-form rather than the mature cytokine as the commercial ELISA kits did not generally distinguish the two proteins, as previously reported (Facci et al., 2014). This hypothesis is also corroborated by the inhibitory effect on LPS-induced increase in IL-1β release from glial cells exhibited by the two inhibitors of p38 and JNK kinases in the MAPK pathway, both involved in the cytokine synthesis inside the cells (Facci et al., 2014).

Since PNP release was slight and IL-1β release was undetectable from neurons, we decided to continue our study focusing on glial cells. Thus, we investigated the activity of ATP, that is liberated from brain cells in normal and even more in pathological brain conditions, including inflammation (Baron et al., 2015). We showed that 500 and 1000 μM ATP, differently from what occurred in rat C6 glioma cells, in which lower ATP concentrations (10 μM) were used, increased PNP release from both astrocytes and microglia after only 1 h from its addition to the cultures. While cell priming for 2 h with LPS did not modify the effect of ATP either on PNP or LDH release from glial cells, it remarkably affected IL-1β secretion, mainly from microglia, as previously reported (Facci et al., 2014).

Regarding the effect of ATP on PNP release, we demonstrated that it occurred mainly through the activation of $P2X_7R$. Indeed, Bz-ATP, an agonist of these receptors, like ATP only at high micromolar concentrations, enhanced PNP release, while, as expected, the $P2X_7R$ antagonist A438079 (Bartlett et al., 2014) blocked the effect induced by the two purine nucleotides. Of course, we cannot rule out that other purine receptors are involved in the effect of ATP. Indeed, we also investigated (data not shown) the activity of a non-selective P2 receptor antagonist, suramin, that reduced the ATP-induced PNP secretion from glial cells, even below control levels. Nonetheless, besides the blockade of ATP activity at any P2 receptor, suramin could induce nonspecific effects, e.g. on calcium release from the endoplasmic reticulum through the stimulation of ryanodine receptors (Hohenegger et al., 1999) or on tyrosine phosphorylation-dependent signaling (Zhang et al., 1998), that may be detrimental for cell functions, as suggested by the finding reported above. Unfortunately, a more selective antagonist against all P2 receptor by a unique molecule is not currently available; therefore, the involvement of other purine receptors will be examined in the future by the use of a great number of antagonists selective for each of the P1 and P2 receptors. Moreover, we cannot also exclude that the enhancement in PNP levels induced by ATP or Bz-ATP could be related to their capability of triggering proliferation of cultured glial cells (Zou et al., 2012).

Finally, our data also indicated a novel aspect in the mechanism leading to PNP secretion from glial cells. It is known that glial cells signal to neighboring cells by releasing molecules *via* regulated exocytosis through lysosomes (Bräuer et al., 2004; Verderio et al., 2012) and that glial cell activation, commonly occurring in most neurodegenerative diseases, affects protein release from these cells by influencing their secretory pathways (Takenouchi et al., 2009b; Bianco et al., 2005; Hanamsagar et al., 2011). Here, we showed that PNP is released by activation of glial lysosomal-secretory pathways, as indicated by the reduction of enzyme activity in conditioned medium from cells following VAMP7 silencing and by PNP localization in LAMP-1-positive vesicles. Interestingly, our data are also in agreement with findings indicating that this non-classical secretory pathway is modulated by ATP through $P2X_7$ receptors in brain immune cells (Dubyak, 2012). In fact, ATP enhanced the amount of PNP and LAMP-1 contained in secreted EVs, which potentially transport proteins, enzymes and RNAs from one cell to another and are key players in intercellular signaling with an emerging role of released vesicles in neuron-glia communication and as support to other glial cells and neurons (Bianco et al., 2005).

5. CONCLUSIONS

In conclusion, our findings demonstrate that brain cells, in particular astrocytes and microglia, not only express, but also release PNP, a key enzyme in the metabolism and the salvage pathway of adenine- and guanine-based purines. In this way, glial cells may support neuronal activity, by maintaining the homeostasis of the purinergic system. In particular, since cerebellar neurons displayed a low content and reduced capability of releasing PNP, we hypothesize that PNP released from glia is particularly important for their function. This hypothesis, which, however, remains to be fully demonstrated, is supported by the evidence that PNP-KO mice show cerebellar abnormalities, including damaged Purkinje cells with degenerated dendrites (Mansouri et al., 2012). We also reported that PNP release from glial cells may be increased by pro-inflammatory agents such as ATP, confirming data about the enhanced PNP expression in peripheral inflammatory

tissues (Batista et al., 2010). Interestingly, PNP release was increased by the interaction of ATP and Bz-ATP with $P2X_7$ receptors, also through an alternative pathway involving lysosomes and used in cytokine secretion (Dubyak, 2012). Noteworthy, these mechanisms may be present in other pathological conditions such as ischemia, traumatic injury and seizure activity (Frühbeis et al., 2013; Hamilton and Attwell, 2010) and would suggest a pathophysiological role for extracellular PNP which needs further investigation. Indeed, increased PNP levels/activity are compatible with the cellular need to control enhanced levels of purines accumulating at extracellular levels as a consequence not only of high amount of extracellular ATP, but also of the stimulatory effect on purine release from cells caused by the nucleotide likely via the interaction with $P2X_7R$ (Franke and Illes, 2014). Whether increased PNP metabolites are harmonically degraded by another key enzyme, possibly present at extracellular levels, such as the xanthine oxidoreductase (XOR) should also be investigated. XOR usually catalyzes the oxidation of hypoxanthine and GUA into xanthine and of xanthine into uric acid, which has antioxidant activity in vivo and represents a defense against free radical formation. However, during tissue damages like those occurring in inflammation, in which there are altered conditions in the extracellular environment (e.g. change in the pH), the activity of XOR may lead to enhanced production of radical species, which are useful for their cytotoxic action against bacteria but may also increase cell/tissue damages (Battelli et al., 2016).

Thus, in this framework, our study on PNP expression, sub-cellular localization, secretion and release regulation in glial cells opens the way to clarify the physiological role of this enzyme and casts light on the pathological mechanisms underlying the modulation of its release/increase.

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure Legends

Fig. 1. PNP expression in rat primary microglia, astrocyte and cerebellar granule neurons (CGNs) cultures. A): PNP mRNA expression in the cultures was analyzed by qRT-PCR. Relative gene expression was calculated using the $\Delta\Delta^{Ct}$ method, where Ct represents the cycle threshold. Δ^{Ct} values were calculated as the difference between the target gene and the expression of the endogenous gene β actin. $\Delta\Delta^{Ct}$ values are the mean ± S.E. of 3 different samples tested in duplicate and are expressed as percentage versus differentiated CGN values. **B and D):** PNP protein expression was analyzed also by Western Blotting in samples (20 μg/lane) of whole homogenates from all cultured cells (B) as well as from microglial cultures after selective glial PNP silencing (D). Each blot was re-probed with an antibody against β-actin to assure equal sample loading. The images in the panels B and D are representative of 5 experiments carried out with different cell samples. Densitometric analysis, reported below each panel, represents the mean ± S.E. of PNP protein expression/β actin *ratio*, then expressed as % *versus* CGNs (B) or *versus* control cultures (D). *p<0.05; **p<0.01: statistical significance as compared to CGNs or control glial cultures (two-

tailed Student's t-test). **C)** Immunofluorescence analysis of PNP expression (green) in rat primary cultures with nuclear Hoechst chromatin staining (blue) (Scale bar: 15 μm)

Fig. 2. Analysis of PNP protein expression and activity in conditioned medium from brain cell primary cultures. (A): Western blot analysis was performed to assay the presence of PNP protein in 500μl concentrated conditioned medium (CM) withdrawn at 1.5, 6 or 24 h after culture medium change from rat primary microglia (M-CM) or astrocyte (A-CM) cultures, both plated at a density of 1.5 × 10^6cells/1ml medium/40mm Ø dish, or neuron cultures (N-CM), plated at a density of 2.4× 10^6cells/1ml medium/40mm Ø dish. Equal loading was confirmed by staining with Red Ponceau S performed on membranes before immunostaining. The panels are representative of three independent experiments, which gave similar results. **(B):** Densitometric values, calculated as PNP/Ponceau staining *ratio*, are the mean±S.E. of three samples. *p<0.05; **p<0.01: statistical significance as compared to values measured at 1.5 h (Student's t test). **(C):** Measurement of PNP activity evaluated in concentrated samples of simplified (Krebs-HEPES buffer) CM removed from cultures of wild type (filled circles) or PNP silenced (open circles) cerebellar neurons (left), astrocytes (middle) or microglial cells (right) 6 h after culture medium change by detection of GUA formation starting from different concentrations of GUO used as enzyme substrate. The enzymatic reaction mixture was incubated for a total of 15 min. The curves with open squares were obtained using medium samples from wild-type cell cultures incubated in a reaction mixture lacking the co-substrate Pi. Values expressed as the mean ± S.E. of three independent experiments were calculated as nmoles of GUA formed from GUO per min and per ml of medium.

Fig. 3. Effect of lypopolyshaccharide (LPS) on PNP, lactate dehydrogenase (LDH) and interleukin-1β (IL-1 β) release from rat cerebellar neurons, astrocytes and microglia.

(A-C) Representative western blot analysis (one of three independent experiments performed) of PNP protein released from rat primary cerebellar neuron (A), astrocyte (B) and microglial (C) cell

cultures stimulated for 24 h with increasing concentrations of LPS (0.1; 0.5; 1 μg/ml). Equal volumes (500 μl) of 24h-conditioned medium from microglia (M-CM), astrocytes (A-CM) and differentiated cerebellar granule neurons (N-CM) were concentrated and loaded for the electrophoretic run. Equal loading was confirmed by staining with Red Ponceau S performed on membranes before immunostaining. **(D-F)**: Evaluation of the release of LDH and Il-1β (on left and right, respectively, in the graph) in the medium from rat brain cells exposed to 1 μg/ml LPS for 24 h. The antagonists of p38 MAPK (SB202190, 10 μM) or JNK (SP600125, 10 μM) were added 1 h prior to cell exposure to LPS. Data are expressed as the mean ± S.E. of three independent experiments. Statistical significance: **p<0.01; ***p< 0.001 compared to controls; #p<0.05 ##p<0.01 compared to LPS-treated cells (two-tailed Student's t-test).

Fig.4. Effect of ATP on PNP, lactate dehydrogenase (LDH) and interleukin 1β (IL-1 β) released from primary rat microglia and astrocyte cultures. (A-B): Western Blot and relative densitometric analyses of PNP protein expression in conditioned medium from primary rat microglia (M-CM) and astrocyte (A-CM) treated for 1 h with increasing ATP concentrations (0, 500 or 1000 μM). Equal loading was confirmed by membrane staining with Red Ponceau S. ATP stimulation was carried out in the presence or absence of a selective $P2X_7R$ antagonist (A438079). Data are expressed as percentage *versus* controls and are the mean ± S.E. from three independent experiments. Statistical significance: *p< 0.05; **p< 0.01 ; ***p<0.001 compared to controls; ##p< 0.01; ###p< 0.001 compared to ATP-treated cells (Dunnett's *post-hoc* comparison test after one-way ANOVA). **(C-D):** Evaluation of the release of LDH and Il-1β in the medium from glial cells primed with 1 μg/ml LPS for 2 h prior to their exposure for 1 h to 500 μM ATP, that was administered either alone or in the presence ofA438079. Data are expressed as the mean±S.E. of three independent experiments. Statistical significance: *p< 0.05; ***p< 0.01 compared to controls; #p<0.05; ##p<0.01 compared to ATP-treated cells (two-tailed Student's t-test).

Fig.5. Effect of BzATP on PNP and interleukin 1β (IL-1β) released from primary rat microglia and astrocyte cultures. (A): Representative images (one of three independent experiments) of PNP protein expression evaluated by Western blot analysis performed on aliquots of 1 h conditioned medium (500 µl) removed from rat cultured microglia (M-CM) and astrocytes (A-CM) treated with increasing concentrations of BzATP (0, 125 or 250µM), in the presence or the absence of the $P2X_7R$ antagonist A438079, added 1 h prior to purine nucleotides. Equal loading was confirmed by staining with Red Ponceau S performed performed on membranes before immunostaining. Data are expressed as the percentage *versus* controls and are the mean ± S.E. from three independent experiments. Statistical significance: ***p< 0.001 compared to controls; ###p< 0.001 compared to BzATP-treated cells (Dunnet's *post-hoc* comparison test after one-way ANOVA). **(B)**: Evaluation of the release of Il-1β in the medium from glial cells exposed to 1µg/ml LPS for 2 h prior to their exposure for 1 h to 125 µM BzATP. Data are expressed as the mean ± S.E. of three independent experiments. Statistical significance: ***p< 0.001 compared to controls; ##p< 0.01 ###p< 0.001 compared to BzATP-treated cells (two-tailed Student's t-test).

Fig. 6. Involvement of VAMP7 and LAMP-1 on PNP release from glial cells.

(A): Immunobands from Western blot analysis and related densitometric evaluation of VAMP27 protein carried out in conditioned medium (CM) from wild type (WT, control) and VAMP7 transiently silenced (siRNA) cells at 16, 24 and 48 h after silencing. β-Actin was used as an internal control to ensure equal sample loading. The panels are representative of three independent experiments, which gave similar results. Densitometric values, calculated as VAMP7/β actin *ratio* and expressed as % of control, are the mean±S.E. of three samples. ***p<0.001: statistical significance as compared to control (Dunnett's post-hoc test was performed after ANOVA). **(B)**: Measurement of PNP activity in samples of simplified (Krebs-HEPES buffer) conditioned media from wild type (WT) cells or VAMP7 silenced cells (siRNA –VAMP), used 16 h after silencing induction, exposed or not to 500 µM ATP (for 1h). Values, expressed as the mean±S.E. of three

independent experiments, were calculated as nmoles of GUA formed from 200 µM GUO per min and per ml of medium. *p< 0.05; **p< 0.01 ***p< 0.01 compared to controls; #p<0.05 ##p< 0.01 compared to ATP-treated cells (two-tailed Student's t-test). **(C):** Co-localization of PNP present in the soluble (SF) and pellet fraction (PF) with the lysosomal protein LAMP-1 isolated from astrocyte and microglia conditioned medim. PNP and LAMP-1 protein expression was evaluated by Western blot analysis performed on aliquots of 2 h-conditioned medium (500µl) collected from rat microglial (M-CM) and astrocyte (A-CM) cultures and on samples from the soluble (SF) or pellet fractions (PF) isolated from the same M-CM and A-CM (as described in the Materials and Methods section) treated or not with ATP (500 µM). Images are representative of three independent experiments giving similar results. Below the blot images the densitomentric analysis of all blots is reported. Each bar in the graph represents the mean ± S.E. of PNP protein expression as percentage *versus* control conditioned medium. **p< 0.01; ***p< 0.01: statistical significance as compared to controls ; #p<0.05 ###p< 0.001 statistical significance as compared to ATP-treated cells (two-tailed Student's t-test).

Supplementary Fig. 1: A representative Western blot for PNP determination in the medium from cultured cells.

Western blot analysis was performed to assay the presence of PNP protein in 500µl concentrated medium (M) withdrawn from two different sets of rat primary microglia (M-CM) or astrocyte (A-CM) cultures, both plated at a density of 1.5 × 10^6cells/1ml medium/40mm Ø dish, or neuron cultures (N-CM), plated at a density of 2.4× 10^6cells/1ml medium/40mm Ø dish, at 6 h after medium change. The blot indicates the selectivity of the antibody against PNP used in this study, able to substantially identify only the band corresponding to the PNP protein, showing a molecular weight of about 32 KDa.

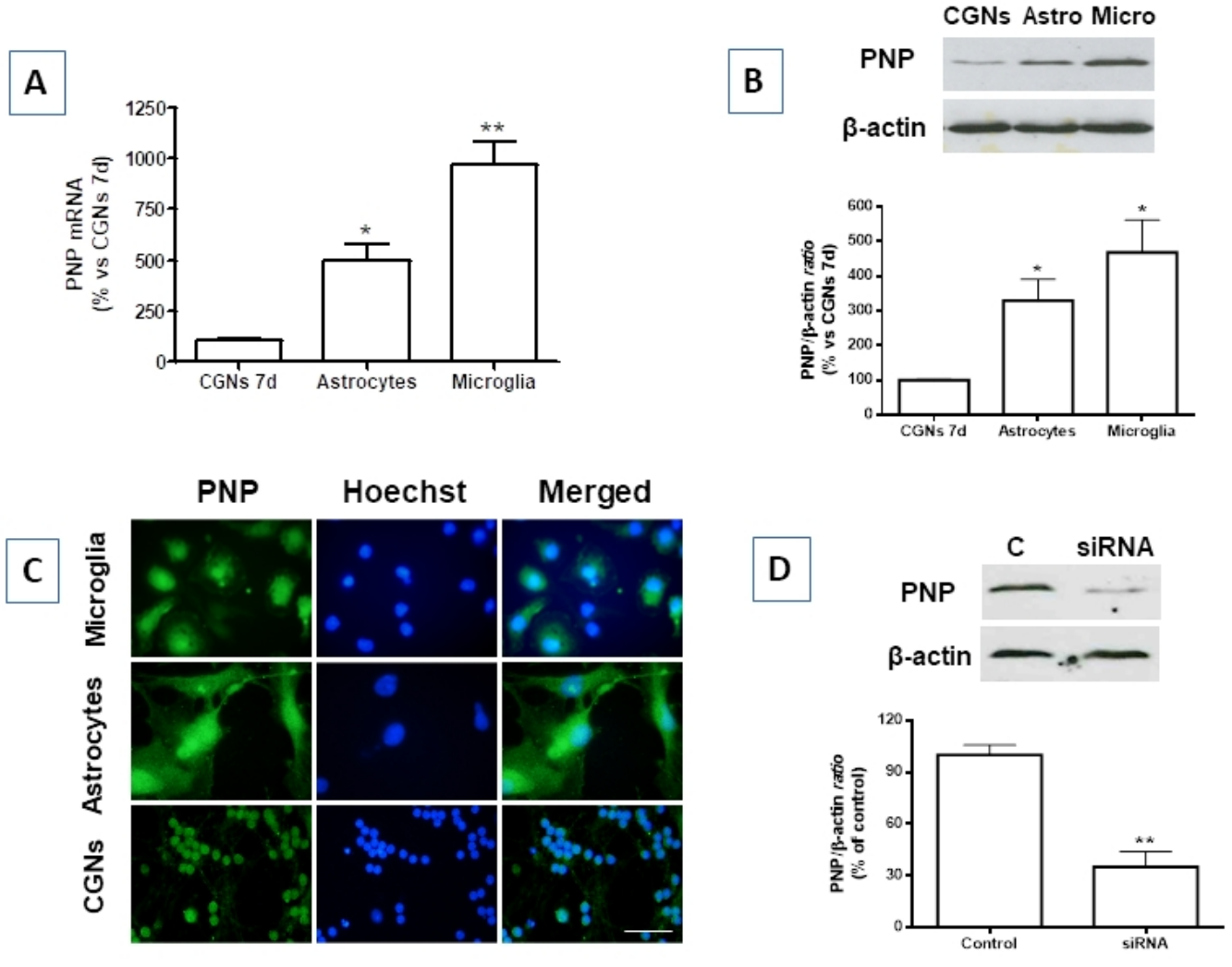

A
PNP mRNA (% vs CGNs 7d)
1250
1000
750
500
250
0
*
**
CGNs 7d
Astrocytes
Microglia
B
CGNs Astro Micro
PNP
β-actin
PNP/β-actin ratio (% vs CGNs 7d)
600
500
400
300
200
100
0
*
*
CGNs 7d
Astrocytes
Microglia
C
PNP
Hoechst
Merged
Microglia
Astrocytes
CGNs
D
C
siRNA
PNP
β-actin
PNP/β-actin ratio (% of control)
120
90
60
30
0
**
Control
siRNA

Figure 1

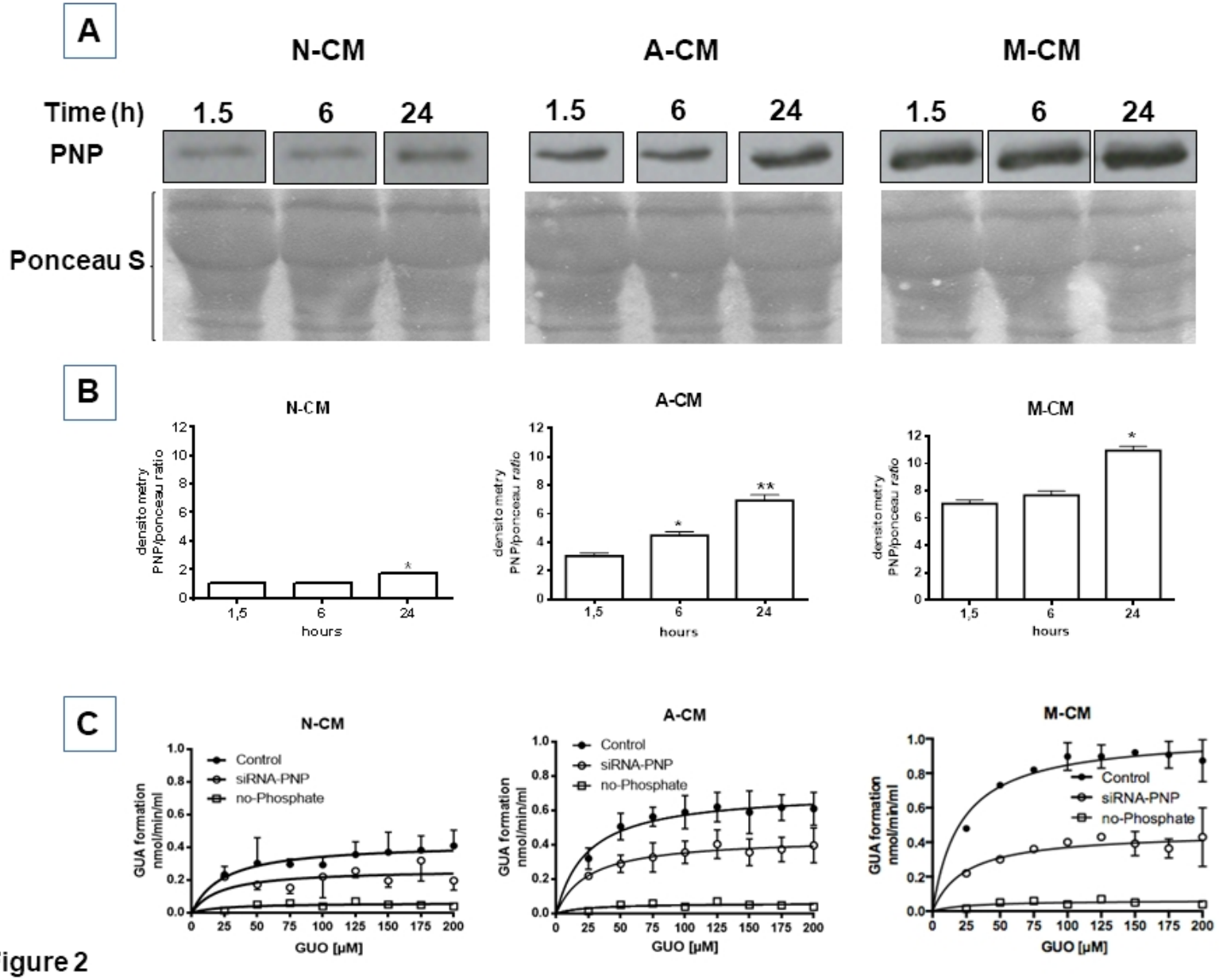

A
N-CM
A-CM
M-CM
Time (h)
1.5
6
24
PNP
Ponceau S
B
N-CM
A-CM
M-CM
densitometry PNP/ponceau ratio
hours
C
N-CM
A-CM
M-CM
Control
siRNA-PNP
no-Phosphate
GUA formation nmol/min/ml
GUO [µM]

Figure 2

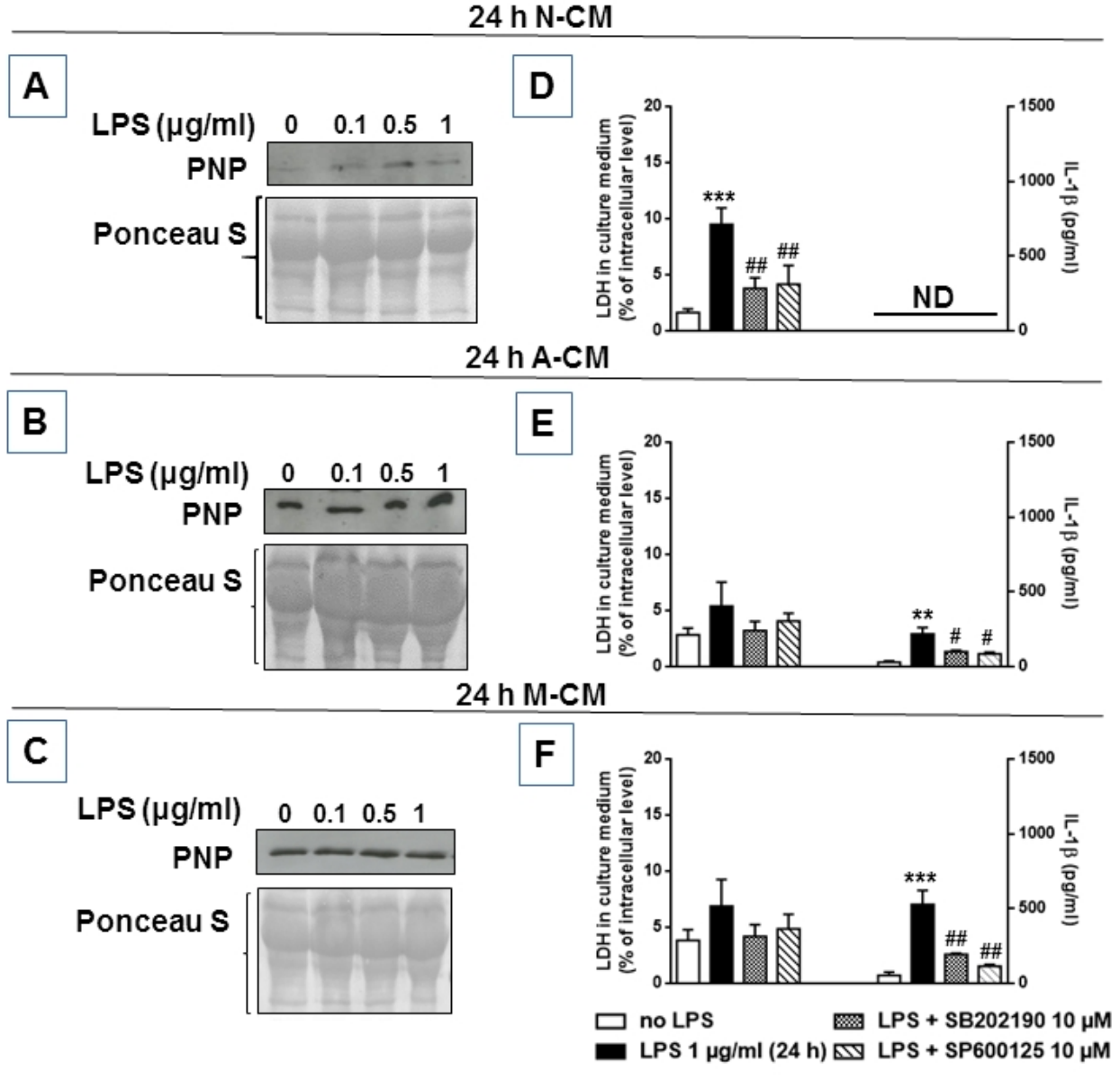

24 h N-CM
A
LPS (µg/ml) 0 0.1 0.5 1
PNP
Ponceau S
D
LDH in culture medium (% of intracellular level)
IL-1β (pg/ml)
***
##
##
ND
24 h A-CM
B
LPS (µg/ml) 0 0.1 0.5 1
PNP
Ponceau S
E
**
#
#
24 h M-CM
C
LPS (µg/ml) 0 0.1 0.5 1
PNP
Ponceau S
F
***
##
##
no LPS
LPS 1 µg/ml (24 h)
LPS + SB202190 10 µM
LPS + SP600125 10 µM

Figure 3

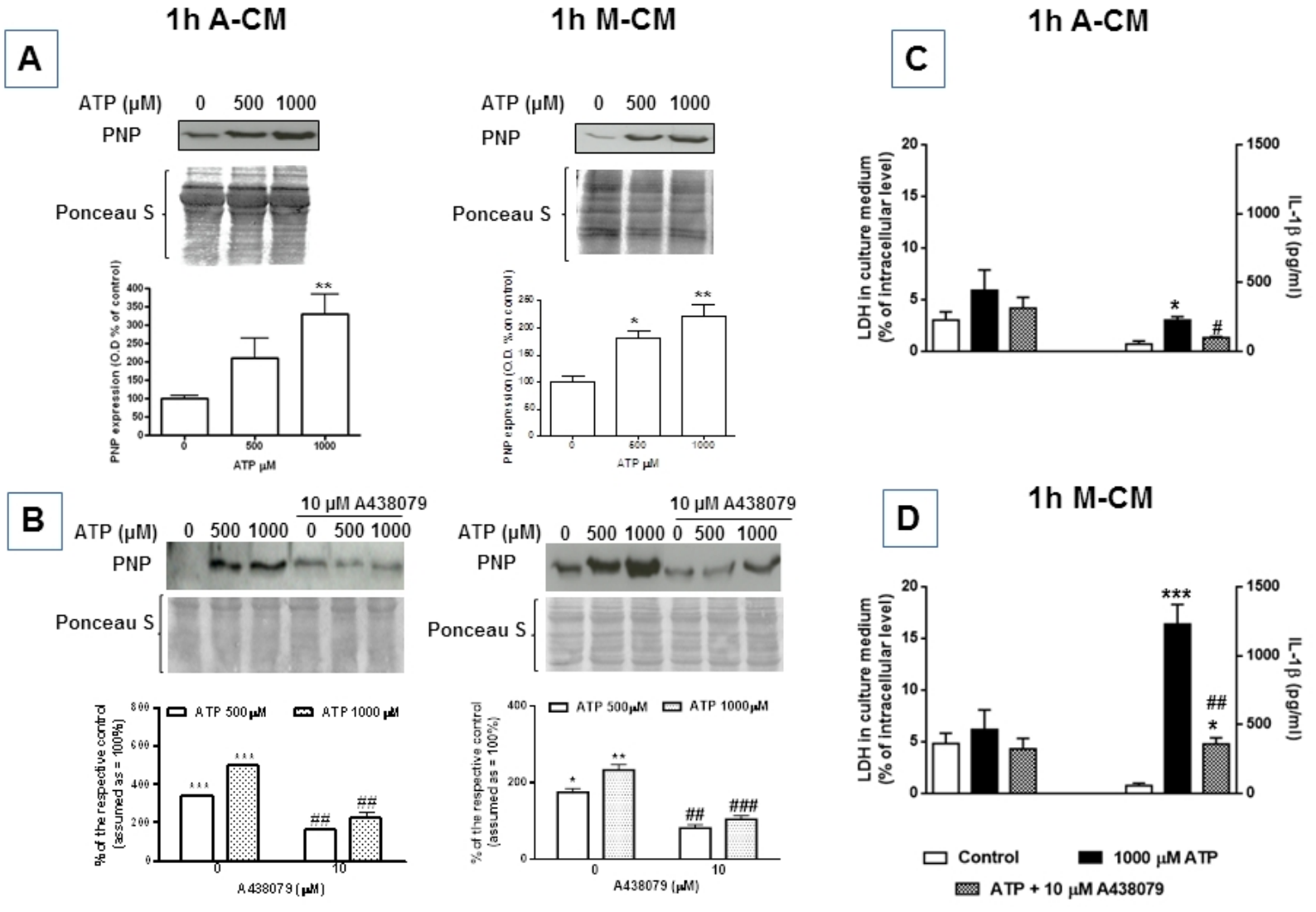

1h A-CM
1h M-CM
A
ATP (μM) 0 500 1000
PNP
Ponceau S
PNP expression (O.D % of control)
ATP μM
B
10 μM A438079
ATP (μM) 0 500 1000 0 500 1000
% of the respective control (assumed as = 100%)
ATP 500μM
ATP 1000μM
A438079 (μM)
C
LDH in culture medium (% of intracellular level)
IL-1β (pg/ml)
D
Control
1000 μM ATP
ATP + 10 μM A438079

Figure 4

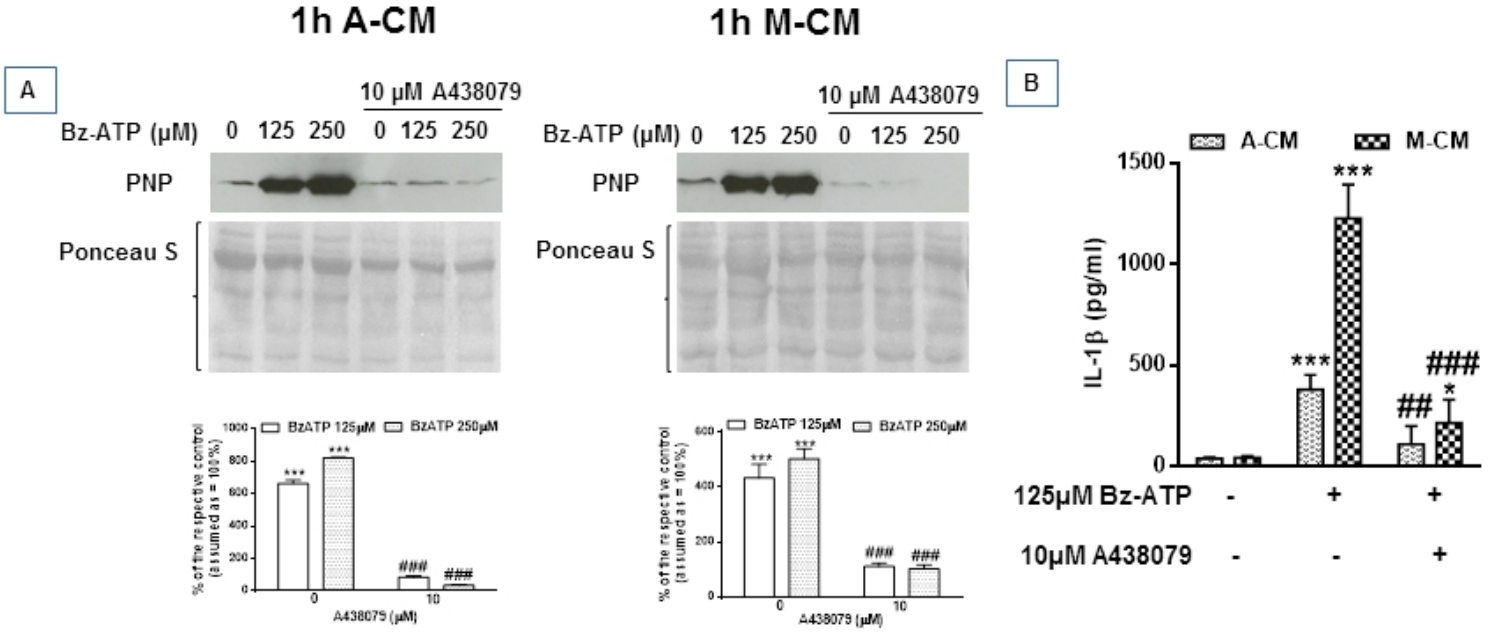

1h A-CM
1h M-CM
A
B
10 μM A438079
Bz-ATP (μM) 0 125 250 0 125 250
PNP
Ponceau S
BzATP 125μM
BzATP 250μM
% of the respective control (assumed as = 100%)
A438079 (μM)
A-CM
M-CM
IL-1β (pg/ml)
125μM Bz-ATP
10μM A438079

Figure 5

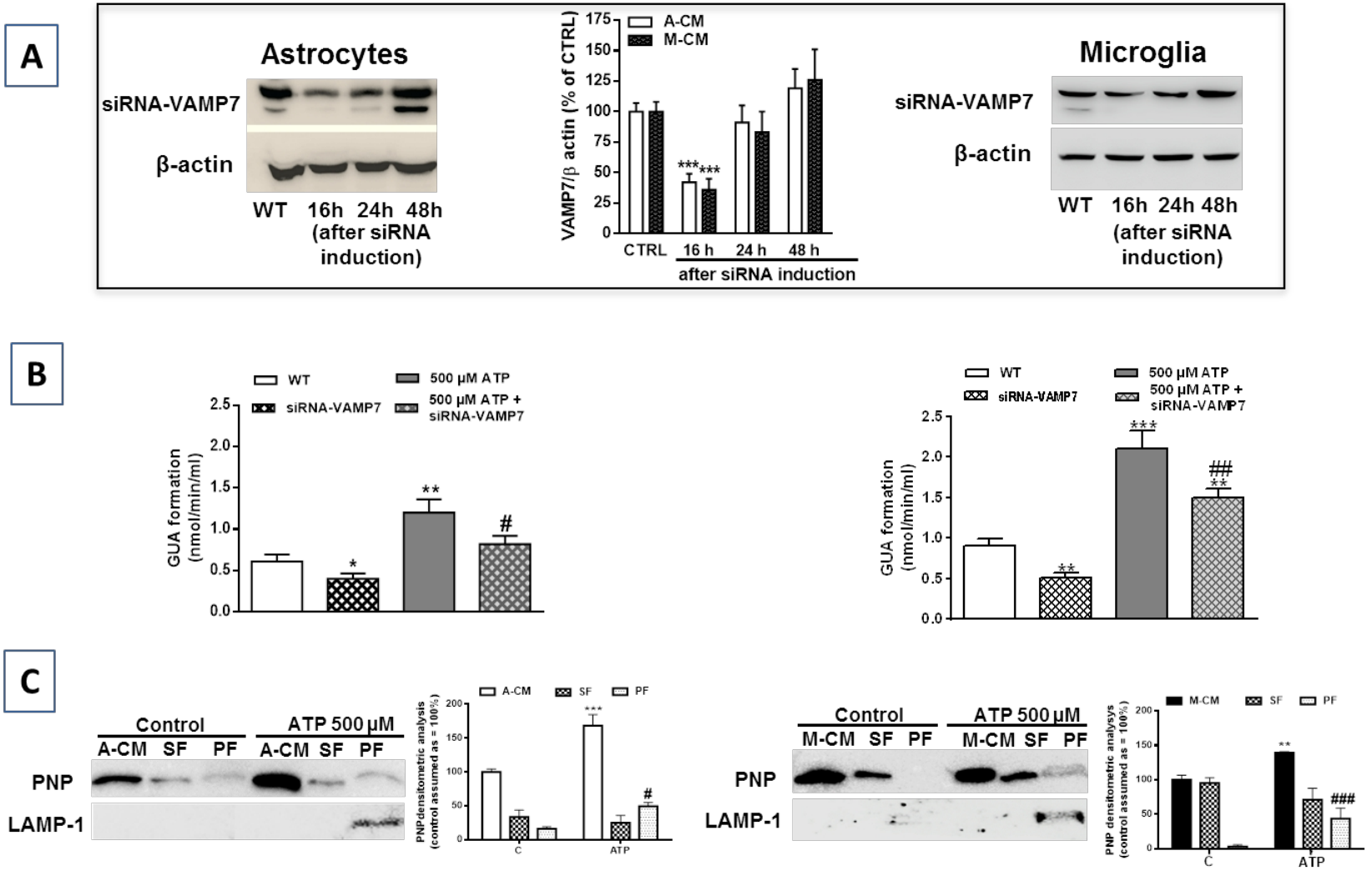
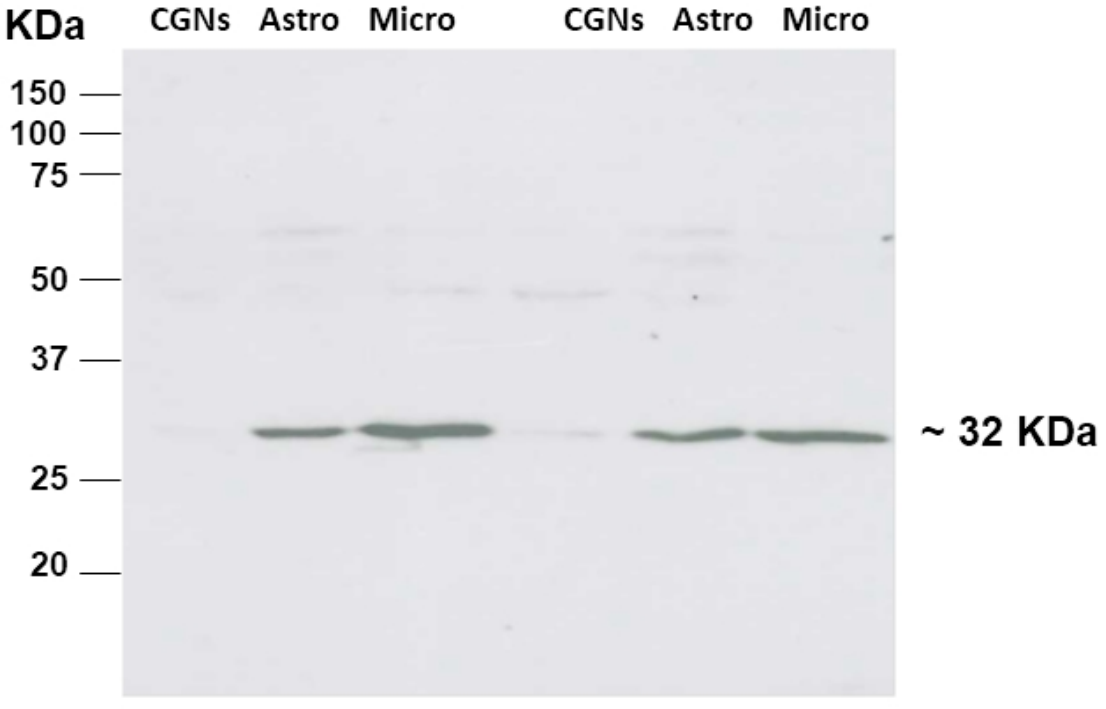

A
Astrocytes
siRNA-VAMP7
β-actin
WT 16h 24h 48h
(after siRNA induction)
A-CM
M-CM
VAMP7/β actin (% of CTRL)
CTRL 16 h 24 h 48 h
after siRNA induction
Microglia
siRNA-VAMP7
β-actin
WT 16h 24h 48h
(after siRNA induction)
B
WT
siRNA-VAMP7
500 µM ATP
500 µM ATP + siRNA-VAMP7
GUA formation (nmol/min/ml)
C
Control
ATP 500 µM
A-CM SF PF
M-CM SF PF
PNP
LAMP-1
PNP densitometric analysis (control assumed as = 100%)
C
ATP

Figure 6



Supplementary Figure 1