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Neuroprotection elicited by $P2Y_{13}$ receptors against genotoxic stress by inducing DUSP2 expression and MAPK signaling recovery

Verónica Morente [a,b,1], Raquel Pérez-Sen [a,b,*,1], Felipe Ortega [c], Jaime Huerta-Cepas [d,2], Esmerilda G. Delicado [a,b], Mª Teresa Miras-Portugal [a,b]

[a] Biochemistry Department, Veterinary Faculty, Complutense University of Madrid, Institute of Neurochemistry (IUIN), Madrid, Spain
[b] Health Research Institute of the Hospital Clinico San Carlos (IdISSC), Spain
[c] Institute of Physiological Chemistry, University Medical Center of the Johannes Gutenberg-Universität Mainz, Germany
[d] Bioinformatics and Genomics Programme, Centre for Genomic Regulation (CRG), Dr. Aiguader, 88., Universitat Pompeu Fabra (UPF), 08003 Barcelona, Spain

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ABSTRACT

Nucleotides activating $P2Y_{13}$ receptors display neuroprotective actions against different apoptotic stimuli in cerebellar granule neurons. In the present study, $P2Y_{13}$ neuroprotection was analyzed in conditions of genotoxic stress. Exposure to cisplatin and UV radiation induced caspase-3-dependent apoptotic cell death, and p38 MAPK signaling de-regulation. Pre-treatment with $P2Y_{13}$ nucleotide agonist, 2methyl-thio-ADP (2MeSADP), restored granule neuron survival and prevented p38 long-lasting activation induced by cytotoxic treatments. Microarray gene expression analysis in 2MeSADP-stimulated cells revealed over-representation of genes related to protein phosphatase activity. Among them, dual-specificity phosphatase-2, DUSP2, was validated as a transcriptional target for $P2Y_{13}$ receptors by QPCR. This effect could explain 2MeSADP ability to dephosphorylate a DUSP2 substrate, p38, reestablishing the inactive form. In addition, cisplatin-induced p38 sustained activation correlated perfectly with progressive reduction in DUSP2 expression. In conclusion, $P2Y_{13}$ receptors regulate DUSP2 expression and contribute to p38 signaling homeostasis and survival in granule neurons.

1. Introduction

Mitogen-activated protein kinases (MAPKs) regulate a great variety of physiological and pathological processes at the central nervous system including survival/apoptosis, proliferation or differentiation. Among them, the extracellular signal-regulated kinases (ERK) are main targets of growth factors and neurotrophins [1–3]. In general, ERK1,2 transient activation mainly trigger neuroprotective actions against apoptotic stimuli [4,5]. Concerning the stress related kinases, p38 and JNK, they mainly function as cell death mediators in response to environmental, inflammatory and danger stimuli [6,7]. Despite this general rule for MAPKs, their real contribution to neural function is not yet totally clarified. In this respect, long-lasting activation of MAPKs, including ERK1/2, is associated to cell death under damaging conditions, such as cytotoxicity, hypoxia/ischemia, oxidative and genotoxic stress [8,9]. Therefore, it is becoming clear that cell fate as a result of MAPK activation seems to be strongly dependent on different factors, such as the cellular environment, type of stimuli and signal duration.

MAPK pathways entail several phosphorylating events that work in cascade. They are tightly regulated at multiple levels, and among them, modulation of protein phosphatase activity plays a key role in the regulation of both duration and magnitude of MAP kinase signaling [10,11]. Different families of protein phosphatases, serine-threonine (PSPs) and tyrosine phosphatase proteins (PTPs and DUSPs), have been identified to operate coordinately to inactivate MAPK signaling, acting through both positive and negative feedback regulatory mechanisms. Among them, dual-specificity protein phosphatases, DUSPs, exhibit activity towards both Thr and Tyr residues. Some members of this family belong to the group of MAPK phosphatases or MKPs, and are emerging as major mediators of the inactivation of sustained MAPK signaling induced by growth factors. Both constitutive and inducible DUSPs can coexist in a cellular model and are transcriptionally regulated by growth

Abbreviations: CaMKII, calcium/calmodulin kinase II; CREB, (cAMP-response-element-binding protein)-binding protein; DAPI, 2-(4-amidinophenyl)-1H-indole-6-carboxamidine; DUSP, dual-specificity phosphatase; ERK, extracellular-signal-regulated kinase; JNK, c-Jun Kinase; 2MeSADP, 2methyl-thio-ADP; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MRS-2179, 2′-Deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt; MRS-2211, 2-[(2-Chloro-5-nitrophenyl) azo]-5-hydroxy-6-methyl-3-[(phosphonooxy) methyl]-4 pyridine carboxaldehyde disodium salt; RT, reverse transcription; NB, Neurobasal-A medium; PI3-K, phosphatidyl inositol-3 kinase; U-0126, 1,4-Diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene

* Corresponding author at: Departamento de Bioquímica y Biología Molecular IV, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda Puerta de Hierro s/n, 28040 Madrid, Spain. Tel.: +34 1 394 38 92; fax: +34 1 394 39 09.
E-mail address: rpsen@vet.ucm.es (R. Pérez-Sen).
[1] These authors equally contributed to the work.
[2] Present address: Structural and Computational Biology Unit, EMBL Heidelberg EMBL Heidelberg, Meyerhofstraße 1, 69117 Heidelberg, Germany.

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factors in a rapid manner as immediately early inducible genes (IEGs) [10,12,13]. They exhibit different substrate selectivity towards a particular MAPK protein, and their expression or activation is normally dependent on the same MAP kinase under regulation [14,15]. Among the most representative ones, DUSP1 and DUSP2 belong to the group of nuclear inducible phosphatases with broad specificity for ERK1/2 and stress kinases, JNK and p38. In addition, DUSP6 belongs to the group of cytoplasmic and constitutive phosphatases more selective for ERK1/2. However, not many pharmacological tools are available to properly characterize protein phosphatases.

Extracellular nucleotides play relevant roles at the nervous system through their coupling to MAP kinase signaling [16,17]. They activate two major families of nucleotide receptors, both ionotropic (P2X) and metabotropic (P2Y) (see recent reviews) [18,19]. In recent works, we described neuroprotective actions displayed by $P2Y_{13}$ and P2X7 receptors in cerebellar granule neurons (CGNs). The survival promoting effect against glutamate excitotoxicity proved to be dependent on the activation of the ERK1/2 target that is the CREB transcription factor. $P2Y_{13}$ and P2X7 receptors were differently coupled to ERK1/2 activation through PI3K and CaMKII-dependent pathways, respectively [20]. In addition, $P2Y_{13}$ receptors prevented oxidative stress-mediated cell death through the up-regulation of the cyto-protective protein heme oxigenase-1 (HO-1), which is a transcriptional product of the master antioxidant regulator Nrf-2 transcription factor [21].

The aim of the present study was to deepen into the meaning of $P2Y_{13}$ receptors neuroprotective actions in granule neurons. Their role was investigated in conditions of genotoxic stress induced by exposure to UV radiation and cisplatin, a drug commonly used in chemotherapy with important neurotoxic side effects [22]. We described the impairment of MAP kinase signaling, especially at the level of p38 phosphorylated form accumulation, in response to cytotoxic treatments. This effect correlated with loss of function of a specific type of dual-specificity protein phosphatase, DUSP2, selective for p38. In addition, activation of $P2Y_{13}$ receptor with 2MeSADP nucleotide agonist was sufficient to prevent p38 over-activation as well as to restore basal DUSP2 expression. Finally, $P2Y_{13}$ activation induced by 2MeSADP contributed to promote neuronal survival against those cytotoxic treatments. Both, the induction of protein phosphatase and the neuroprotective effect were dependent on PI3K/ERK-dependent signaling triggered by $P2Y_{13}$ receptors in granule neurons [20].

2. Materials and methods

2.1. Antibodies and materials

Nucleotide receptor agonists and antagonists, and transducing inhibitors, 2MeSADP and ortho-vanadate, were purchased from Sigma Aldrich (St Louis, USA), MRS-2211 from Tocris Bioscience (Essex, UK), and Wortmannin and U-0126, from Calbiochem Co. (San Diego, USA). Specific antibodies for phosphor-ERK1/2 (Tyr204), ERK2 were from mouse, and for DUSP2 were from goat, and purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific for GAPDH, 17 kDa-Caspase-3 fragment, phosphor-p38, phosphor-JNK and p38 were from rabbit and purchased from Cell Signalling Technology (Beverly, MA, USA). Secondary horseradish peroxidase-conjugated antibodies were from Santa Cruz Biotechnology (anti-mouse), and Dako (Denmark) (anti-rabbit, anti-goat) and Synaptic Systems (donkey anti-rabbit IgG Cy3-conjugated). All other reagents not specified were routinely supplied by Sigma, Merck (Darmstadt, Germany) or Roche Diagnostics SL (Barcelona, Spain).

2.2. Cell culture and treatment conditions

All experiments carried out at the Universidad Complutense de Madrid followed the guidelines of the International Council for Laboratory Animal Science (ICLAS). Cerebellar cultures were performed according to the procedure described by Pons et al. with some modifications [23]. Cerebella from Wistar rat pups (P7) were aseptically removed, and submitted to digestion with papain 100 U/ml (Worthington, Lake Wood, NJ) (previously activated in EBSS buffer containing 5 mM L-Cys and 2 mM EDTA), in the presence of 100 U/ml of DNAse (Worthington, Lake Wood, NJ), 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The obtained cells were resuspended in neurobasal medium supplemented with B-27 (GIBCO, BRL, Paisley, Renfrewshire, UK) containing 21 mM KCl, 2 mM glutamine, and antibiotics, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B (Sigma Aldrich, St Louis, USA), and plated onto glass coverslips or plastic Petri dishes (60 and 35 mm) (Falcon Becton Dickinson Labware, Franklin Lakes, USA) precoated with 0.1 mg/ml poly-L-lysine (Biochrom, AG, Berlin) at a density of 200,000 cells/cm^2. They were maintained in a humidified incubator at 37 °C in 5% CO_2. AraC (10 μM) was added to avoid the proliferation of glial cells.

Cultured cerebellar granule neurons were used at 8–10 DIV (days in vitro), the stimulation of cells with the nucleotide agonists or other agents was carried out adding the corresponding compounds to cells maintained in complete culture media. These stimulations were done at specified times before the addition of the cytotoxic drug, cisplatin at concentrations of 20 or 40 μg/ml. The exposition to UV radiation was performed for 2 min under transilluminator lamp (32 J/cm^2), after that, cells were maintained in the incubator until the times required.

2.3. Western blot experiments

In phosphorylation assays of MAPK proteins, cell extracts or lysates were obtained at different incubation periods. Stimulation was stopped by the addition of lysis buffer (20 mM MOPS, 50 mM NaF, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM EDTA, 2 mM EGTA, 0.5% Triton X-100, pH = 7.2, 1 mM PMSF and protease inhibitor cocktail (Complete, Roche)). Protein determination of the cell extracts was performed and then mixed with sample buffer 4× (50% glycerol, 125 mM Tris pH 6.8, 4% SDS, 1% bromophenol blue, 5% β-mercaptoethanol, 4.5% H_2O). The samples were heated to 99 °C and aliquots (25 μg) were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis (25 mM Tris, 200 mM glycine, 0.1% SDS, pH = 8.3) using 12% acrylamide gels. Immunotransference was performed in PVDF membranes (Amersham Biosciences Europe GmbH, Barcelona, Spain) (25 mM Tris, 192 mM glycine, 20% methanol). The TBS buffer (100 mM NaCl, 10 mM Tris–HCl, pH 7.5) containing 1% (v/v) Tween-20 and 5% BSA was employed as blocking medium and in subsequent incubations with the antibodies. Incubation with the antibodies was performed at the following dilutions: 1:1000 for phosphor-ERK1/2, total ERK (Santa Cruz), total p38, GAPDH, and caspase-3 (Cell Signalling), and 1:500 for phosphor-p38, phosphor-JNK (Cell Signalling) and DUSP2 (Santa Cruz). Primary antibodies were detected with horseradish peroxidase-conjugated antibodies, 1:4000 for anti-mouse (Santa Cruz) and anti-rabbit (Dako), and 1:2000 for anti-goat (Santa Cruz) visualized by the ECL method (kit Super Signal substrate Western Blotting, from Amersham Biosciences Europe GmbH, Barcelona, Spain). The chemiluminescence images were quantified by densitometry employing the Fluo-S Imager of Bio-Rad (Munich, Germany).

2.4. Microarray analysis

RNA was collected from cells treated with 1 μM 2MeSADP for 2 h. Samples were hybridized with a chip of genomic expression Agilent, capable of quantifying the expression of 28,000 rat genes. 4 replicates of each condition (Control or 2MeSADP) were performed and analyzed. The resultant expression data was normalized following the method described by Bolstad et al. [24] and implemented into the analysis package affy [25] for the statistic environment R (GNU project http://www.r-project.org/).

T-test was employed to obtain expression differences between control and treated samples. *T*-test following subsequent correction by Bonferroni method was applied to the average expression of each gene within the two groups. To achieve that, the analysis platform for microarrays GEPAS [26] was employed along with the statistic environment R.

Functional analysis of the genes, sorted by their degree of differential expression, was performed employing the bioinformatics software Fatiscan [27], currently implemented into the online platform Babelomics [28]. This method allows, by the employing of a segmentation test, to detect functional groups of genes that additionally exhibit expression differences. A short partition size of 50 genes, a two-tails Fisher test and a significant degree of significance of $P < 0.05$ was used in this analysis.

2.5. Real time RT-QPCR

Total RNA was isolated from granule cells after the indicated treatments for different periods of time with the RNeasy Qiagen kit, and 1 μg DNAse-treated RNA (Turbo-DNA free, Ambion) was used for first strand cDNA synthesis, according to the manufacturer's protocol (TaqMan Reverse Transcript Reagents, Applied Biosystems kit). Quantitative real time PCR was performed using Applied Biosystems Step One Plus and specific primer and probe sets were purchased from Roche Applied Science (Universal ProbeLibrary Probes labeled with FAM), being the forward and reverse primer pairs: AGGCTATCGGCTTCATTGAC/TCGATGGCTCTGAATCAGGT for *dusp2*, and CCCCTCTGGAAAGCTGTG/GGATGCAGGGATGATGTTCT for *gapdh*. The amplification was performed using LuminoCt® Qpcr Ready Mix™ (Sigma) and run for 20 cycles (94 °C 30 s, 60 °C 30 s). Relative quantifications were normalized with respect to the *gapdh* endogenous control data, and results are expressed as increments in expression with respect to control conditions (in the absence of any stimulation).

2.6. Immunocytochemistry experiments

Cells plated onto glass coverslips were fixed with PFA (4%, w/v) for 15 min and permeabilized with Triton X-100 (0.1%, v/v) in PBS/BSA (3%, w/v) containing 5% of donkey serum for 1 h at 37 °C. The primary antibody, rabbit anti-phosphor-p38 (1:100) was diluted in PBS/BSA and applied overnight at 4 °C. The secondary antibody, donkey anti-rabbit IgG Cy3-conjugated (1:400), was incubated in PBS/BSA for 1 h

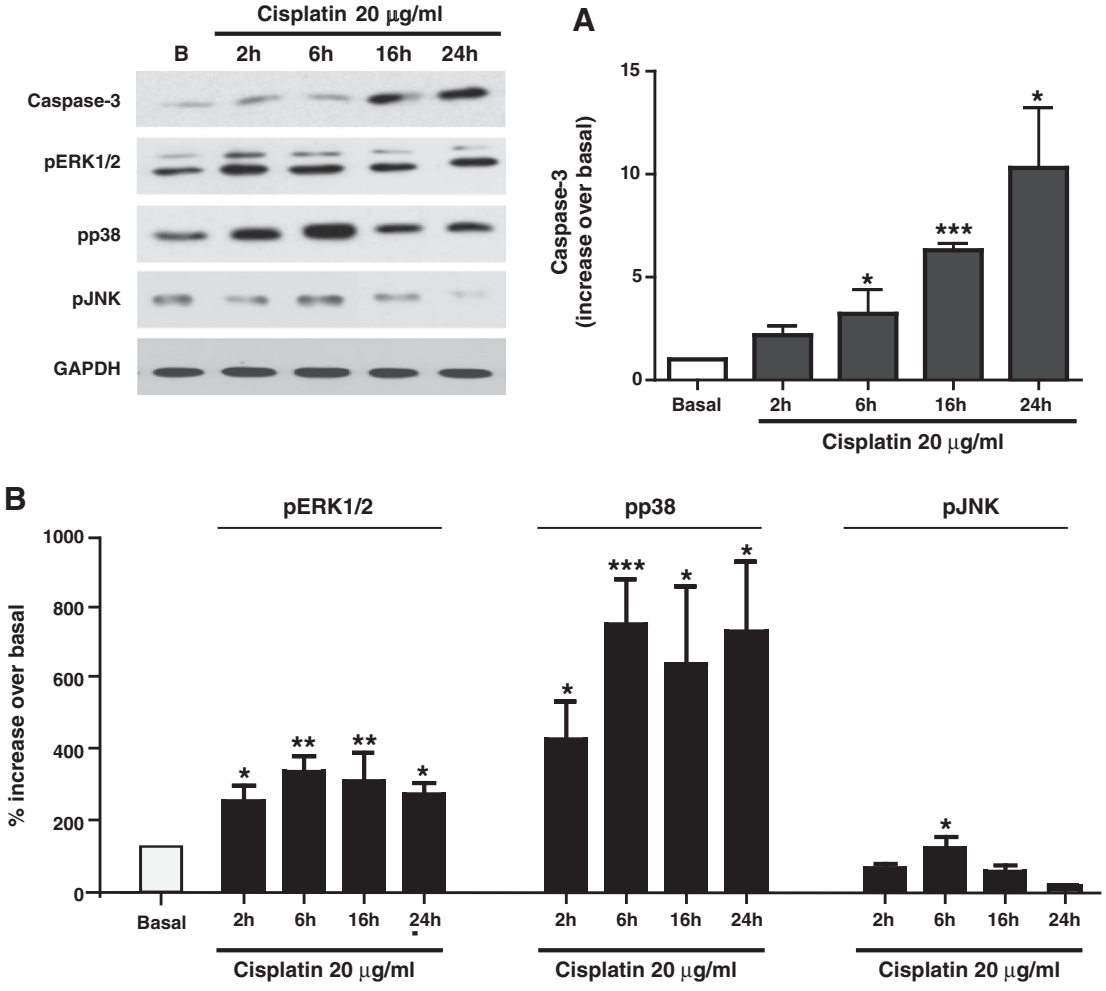

Fig. 1. Activation of caspase-3 and MAPK proteins upon cisplatin treatment in granule neurons. Granule neurons in complete culture media were exposed to 20 μg/ml cisplatin and total cell lysates were collected at different times after treatment. Analysis was carried out by immunoblot using antibodies against the 17 kDa active fragment of caspase-3 (A) and the phosphorylated forms of ERK1/2, p38 and JNK (B), as described in the Materials and methods. The blots correspond to representative experiments. Histograms represent the increase with respect to non-stimulated cells (100% basal), and were obtained by normalization of densitometric values of phosphoproteins with respect to GAPDH. Values are the means ± SD of experiments performed from different cultures (n = 10). Data were statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, when the effects after cisplatin treatment (solid bars) were compared to basal (open bars, in the absence stimulation).

at 37 °C. Finally, cells were washed three times and incubated with 1 μM DAPI to stain nuclei. After two washes in PBS, covers were mounted following standard procedures (Prolong Anti-Fade). Controls were prepared according to the same protocol by replacing primary antibodies with the same volume of PBS/BSA solution, and followed by the incubation with the secondary antibodies. Confocal images were acquired with a TCS SPE microscope from Leica Microsystems (Wetzlar, Germany). Densitometric analysis of the photographs was performed using ImageJ and Leica LAS AF Lite software.

2.7. Cell viability assays

Cells were treated for 1–2 h with the nucleotide agonist, 2MeSADP, in the absence or presence of nucleotide receptor antagonist or transducing protein inhibitors, before the addition of cisplatin (20 μg/ml), or the exposure to UV radiation for 2 min. Cell viability was tested 24 h later by the MTT assay that measures mitochondrial function. After the corresponding treatments, the tetrasodium salt MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) was added to the cultures to a final concentration of 0.5 mg/ml, and maintained for 2 h at 37 °C. Then, an equal volume of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added, following a brief incubation of 30 min at room temperature with orbital shaking. The samples were collected and measured spectrophotometrically at 570 nm. Values were normalized in respect to that obtained from untreated cells, considered as 100% survival.

2.8. Statistical analysis

Data are represented as means ± SD of at least 3 independent experiments obtained from different cultures. Comparison between different treatments and controls was carried out using Dunnett's test, and comparison between different samples was done by applying Tukey's test.

3. Results

3.1. MAPK signaling is deregulated in granule neurons upon cisplatin exposure

MAPK signaling cascades result deregulated in response to several toxic insults that induce genotoxic stress. We studied these alterations in granule neurons exposed to the genotoxic agent, cisplatin. Exposure

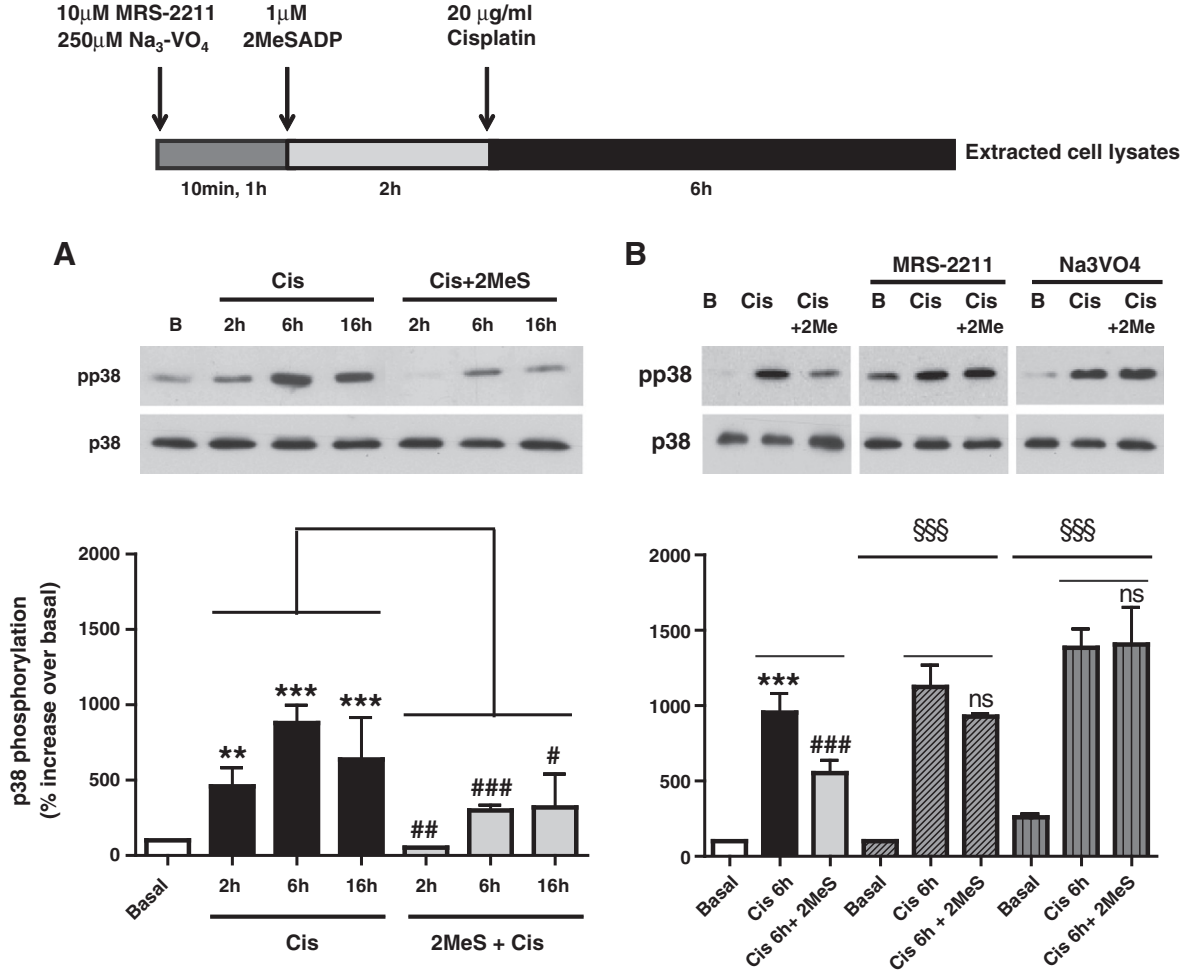

Fig. 2. Effect of $P2Y_{13}$ nucleotide agonist on cisplatin-induced p38 sustained activation in cerebellar granule neurons. (A) Granule neurons were incubated in the presence or absence of 1 μM 2MeSADP for 2 h, before the addition of 20 μg/ml of cisplatin, and then phosphorylated p38 was analyzed by immunoblot in cell lysates collected at the indicated times. (B) Granule neurons were incubated in the presence or absence of the $P2Y_{13}$ antagonist 10 μM MRS-2211 for 10 min, or the tyrosine phosphatase inhibitor, 250 μM orthovanadate, for 1 h, before the nucleotide agonist. 2 h later cisplatin was added and cell lysates were collected at 6 h. The blots correspond to representative experiments. Histograms represent the percentage increase with respect to non-stimulated cells (100% basal), and were obtained by normalization of densitometric values of phosphor-p38 with respect to total p38. Values are the means ± SD of experiments performed from different cultures (n = 9). Data were statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, when the effects of cisplatin treatment were compared to basal (open bars, in the absence stimulation), and at $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, when each cisplatin incubation time was compared in the absence or presence of 2MeSADP. In (B) data were statistically significant at §§§$P < 0.001$ when the treatments were compared in the presence or absence of antagonist and inhibitor. The upper picture in the figure shows the experimental design.

to this drug is known to be cytotoxic for different cell types [22], and accordingly to this, a slow accumulation of the 17-kDa caspase-3 active fragment was observed over time, being maximal after 16–24 h treatment and indicating the activation of apoptotic cell death pathway in granule neurons (Fig. 1A). MAP kinase signaling was then analyzed at different times after cisplatin administration by measuring the phosphorylated forms of ERK1/2, p38 and JNK. Exposure to cisplatin caused the sustained phosphorylation of both ERK1/2 and p38 proteins. For

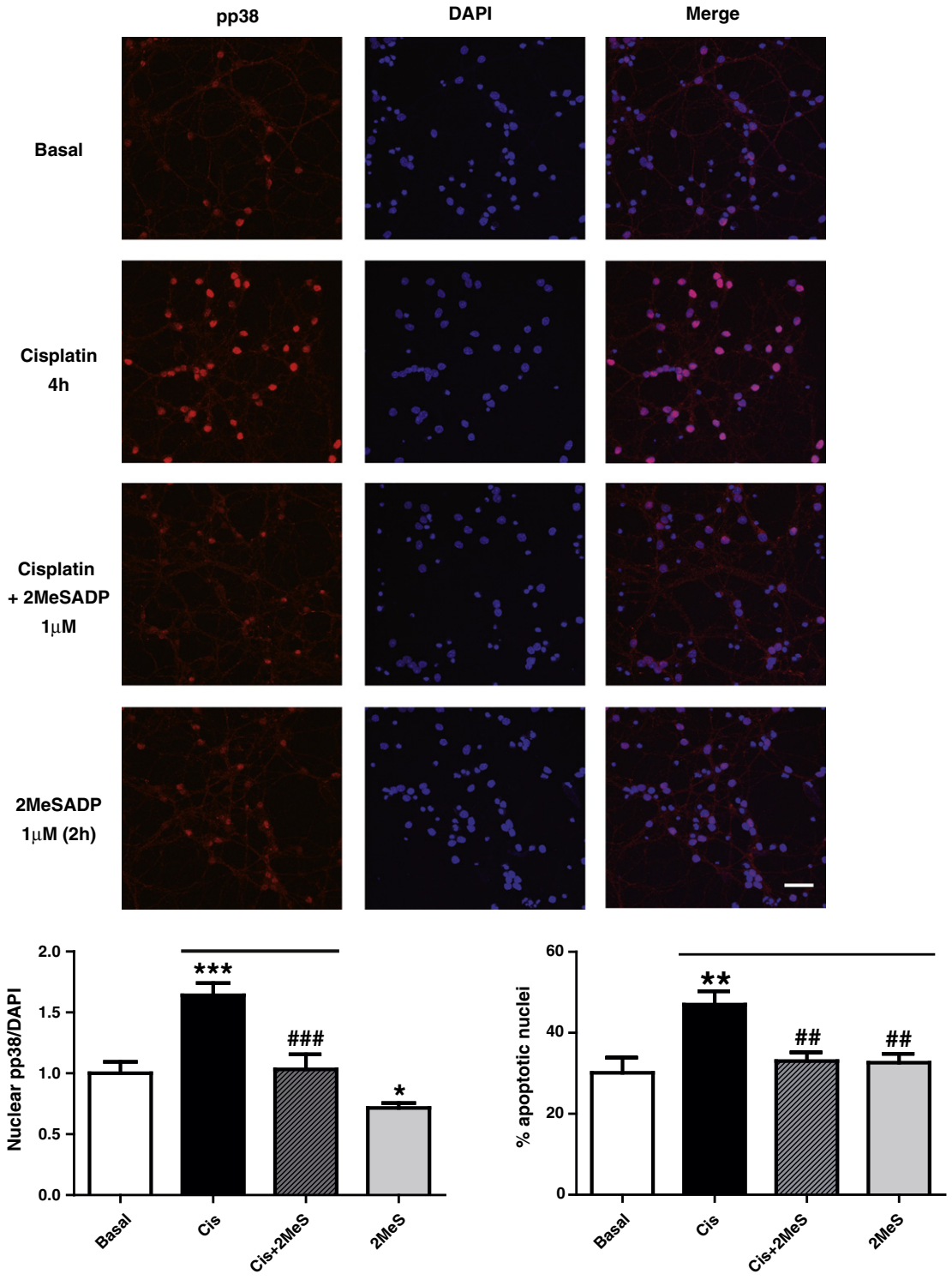

Fig. 3. Nuclear accumulation of phosphorylated p38 form upon cisplatin treatment in granule neurons. Granule neurons were incubated in the presence or absence of 1 μM 2MeSADP for 2 h, before the addition of 20 μg/ml of cisplatin, and then phosphorylated p38 was analyzed by immunochemistry as described in the Materials and methods. Confocal images were analyzed for colocalization between phosphor-p38 and DAPI signals and quantification of nuclear phosphorylated p38 accumulation was performed by Leica LAS AF Lite software. Scale bar corresponds to 50 um. Histograms in left graph represent the ratio between nuclear phosphorylated p38 and DAPI intensity signal in each field and normalized with respect to control. Right graph represents quantification of nuclei exhibiting apoptotic-like morphology. Values are the means ± SD of experiments performed from different cultures (n = 4). Data were statistically significant at $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ when the effects were compared to basal (open bars, in the absence stimulation), and at $\#\#\#P < 0.001$ and $\#\#P < 0.01$ when cisplatin treatment was compared in the absence or presence of 2MeSADP.

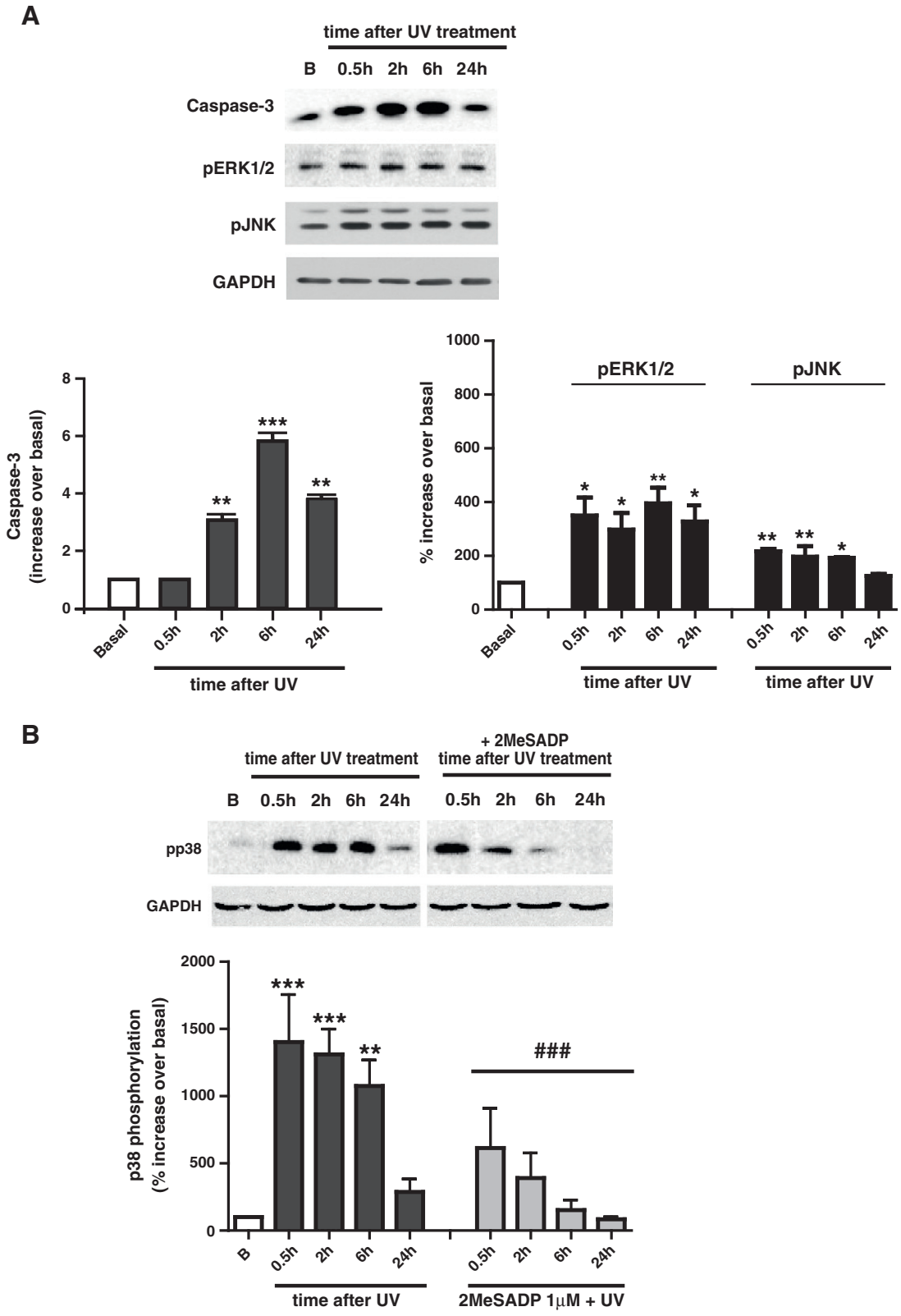

Fig. 4. Activation of caspase-3 and MAPK proteins upon UV treatment in granule neurons. Effect of 2MeSADP nucleotide agonist. Cells were exposed to UV for 2 min, and total lysates were collected at different times, as indicated. Analysis was carried out by immunoblot using antibodies against caspase-3 and the phosphorylated forms of ERK1/2, JNK (A) and p38 (B), as described in Methods. In B, granule neurons were pre-treated in the presence or absence of 1 μM 2MeSADP for 2 h before UV exposure. The blots correspond to representative experiments. Histograms represent the percentage increase with respect to non-stimulated cells (100% basal), and were obtained by normalization of densitometric values of the studied proteins with respect to GAPDH. Values are the means ± SD of experiments performed from different cultures (n = 6). Data were statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, when the effects after UV treatment (solid bars) were compared to basal (open bars, in the absence stimulation), and at $\#\#\#P < 0.001$, when the effect at each incubation time was compared in the absence (solid bars) or presence of 2MeSADP (gray bars).

both MAPKs, maximal phosphorylation levels were reached after 6 h. Changes were most significant for p38, which resulted an increase of about 8–10 times over basal levels, whereas moderate levels of around 2–3 times were obtained for ERK1/2 protein phosphorylation (Fig. 1B). In contrast, phosphorylated levels were barely affected for the other stress-related MAP kinase, JNK (Fig. 1B).

3.2. $P2Y_{13}$ nucleotide receptor agonist, 2MeSADP, prevents cisplatin-induced p38 sustained phosphorylation

The effect of $P2Y_{13}$ nucleotide receptor activation under cisplatin treatment was then analyzed with respect to p38 signaling over-activation. According to that previously described, the nucleotide agonist 2MeSADP was employed to activate $P2Y_{13}$ receptors expressed in granule cells [20,29,30]. Treatment with 1 μM 2MeSADP for 2 h prior to cisplatin exposure was sufficient to reduce long-lasting levels of p38 phosphorylation in a significant percentage, which accounted for 50–60% decrease at different times of treatment with the cytotoxic drug (Fig. 2A). Several incubation times with the nucleotide agonist were tested, and the dephosphorylation effect began to be evident from the first hour of treatment, without apparent improvements if it was prolonged for 4 h. Therefore, 2 h pre-incubation time was chosen to get the most significant effect, fitting perfectly to the previous work in which 2MeSADP mediated neuroprotective effects were observed at this incubation period [20]. Under these conditions, pre-treatment with $P2Y_{13}$ antagonist, MRS-2211, totally abolished the ability of 2MeSADP to decrease p38 phosphorylation levels, elevated as a consequence of cisplatin treatment, confirming the specificity of the effect (Fig. 2B).

These results suggested that the mechanism of action of $P2Y_{13}$ nucleotide agonist preventing p38 MAPK over-activation in granule neurons could require a protein phosphatase activity. This was supported by using orthovanadate, a general inhibitor of protein tyrosine phosphatases. Pretreatment of granule neurons with this inhibitor did not induce a significant change in basal levels of phosphorylated p38 proteins, although slightly increased when obtained in the presence of cisplatin. Noticeably, it prevented the ability of 2MeSADP to reduce sustained p38 phosphorylation (Fig. 2B).

MAPK signaling elicited by $P2Y_{13}$ receptor activation was also analyzed as a function of time in granule neurons. 2MeSADP was coupled to ERK1/2 phosphorylation in a transient pattern, reaching a peak at 30 min of exposition time and slowly returning towards baseline levels at 1–2 h (Supp. Fig. 1A). In contrast, no changes in p38 phosphorylation levels were detected upon treatment with 2MeSADP, indicating that $P2Y_{13}$ receptors are not coupled to p38 activation in this cellular model (Supp. Fig. 1B).

3.3. $P2Y_{13}$ nucleotide agonist, 2MeSADP, prevents phosphorylated p38 nuclear accumulation induced by cisplatin treatment

Considering that several danger stimuli evoke translocation of active p38 form from cytoplasm to the nucleus [31], p38 subcellular distribution was analyzed by immunocytochemistry in granule neurons following cisplatin exposure. In accordance to that described in neural models, basal phosphorylated p38 levels were very low and uniformly distributed throughout the nucleus and cytoplasm in non-stimulated cells, showing slight decreases upon stimulation with 2MeSADP for 2 h. After 4 h cisplatin treatment, an intense staining pattern was observed for phosphorylated p38 at the nucleus co-localizing perfectly with nuclear binding dye, DAPI. As expected, 2 h pre-treatment with 2MeSADP exhibited a robust effect reversing cisplatin-induced p38 nuclear accumulation, and phosphor-p38 levels returned to basal levels (Fig. 3). In addition, 2MeSADP was able to prevent increase in apoptotic nuclei observed after cisplatin treatment.

3.4. $P2Y_{13}$ nucleotide agonist, 2MeSADP, prevents UV-induced p38 sustained activation

UV radiation is another approach that was analyzed to induce genotoxic stress conditions. Granule cells were exposed to UV radiation, and then cell extracts were collected at different times. In comparison to cisplatin treatment, UV light resulted to be a more potent apoptotic stimulus, as caspase-3 activation was reached at earlier incubation time of 6 h (Fig. 4A). In addition, UV radiation caused similar effects to cisplatin treatment in terms of long-lasting MAPK protein phosphorylation. Again, major increases were observed at the level of p38 protein that reached 10-fold as early as 30 min after UV exposure and decreasing only at 24 h. Minor 2-fold increments were obtained for both ERK1/2 and JNK proteins (Fig. 4A and B). As expected, pre-treatment with 1 μM 2MeSADP for 2 h prior to UV exposure, was able to decrease significantly p38 phosphorylated levels at all times tested, in a similar manner to that observed with cisplatin (Fig. 4B). These results indicate that p38 activation appears to play a central role in granule neurons submitted to genotoxic stimuli, and that $P2Y_{13}$ receptors seem to operate through similar intracellular mechanisms in restoring basal p38 MAPK phosphorylation.

3.5. Microarray gene expression analysis in granule neurons stimulated with 2MeSADP

The above results provide evidence of $P2Y_{13}$ involvement in the regulation of protein phosphatase activity, restoring basal p38 phosphorylation after exposure to cisplatin or UV light. Different kinds of tyrosine protein phosphatases exhibiting substrate specificity towards MAPKs

Table 1
Functional enrichment analysis of rat genes in control and 2MeSADP treated cultures of cerebellar granule neurons.

Gene Ontology term	GO term	#genes (UpList)	#genes (OutList)	Genes present in the up-regulated list	Adj. P value
Protein dephosphorylation	GO:0006470	98	7	Dusp13, Dusp9, Ptprj, Sox10, Sox5, Ptpn18, **Dusp2**, Ptpn6, (…)	1.66E−002
Cellular protein modification process	GO:0006464	767	87	Bcam, Prkci, Sox5, Thy1, Arrb1, Il2, Ptpn18, **Dusp2**, Stk17b (…)	9.84E−009
Dephosphorylation	GO:0016311	116	9	Dusp13, Dusp9, Ets1, Ptprj, Pnkp, **Dusp2**, Ptpn6, (…)	1. 32E−002
Post-translational protein modification	GO:0043687	673	81	**Dusp2**, Il1b, Ripk3, Dusp13,Dusp9, Arrb1, Il2, Ptpn18, (…)	1. 00E−006
Phosphate-containing compound metabolic process	GO:0006796	686	79	**Dusp2**, Sox10, Kalrn, Bard1, Bcam, Prkci, Sox5, Thy1, Arrb1, (…)	1.14E−007
Enzyme binding	GO:0019899	237	64	Thy1, **Dusp2**, Plce1, Serpine1, Akap14, Pawr, Tgfbr2, Gck, Insr, (…)	1.12E−003
Hydrolase activity, acting on ester bonds	GO:0016788	331	39	Dusp13, **Dusp2**, Plcb3, Dusp9, Aste1, Pola1, Ets1, Aspa, Usp16, (…)	1. 84E−003
Phosphatase activity	GO:0016791	150	14	Dusp13, Dusp9, Ets1, Ptprj, Sox10, Sox5, Pnkp, **Dusp2**, Ppap2, (…)	1. 98E−002
Phosphoprotein phosphatase activity	GO:0004721	104	6	Dusp13, Dusp9, Ets1, Ptprj, Sox10, Sox5, **Dusp2**, Ptpn, (…)	5.84E−003
Protein kinase binding	GO:0019901	105	3	Akap14, Tgfbr2, Insr, Ets1, Cdk5rap2, Sox10, **Dusp2**, Prkab2, (…)	1. 00E−004
Kinase binding	GO:0019900	119	6	Akap14, Tgfbr2, Insr, Ets1, Cdk5rap2, Sox10, **Dusp2**, Prkab2, (…)	7.69E−004

Genes present in several significantly up-regulated functional Gene Ontology (GO) categories. The software FatiScan [27], as implemented in the babelomics web tool [27], was used to analyze the complete list of rat genes ranked according to their differential expression (*T*-test) between control and 2MeSADP treated cultures. For each functional term, *UpList* refers to the number of genes in a functional category that exhibit higher expression differences than the rest in the same category (Out*List*). Note that Dusp2 appears associated to the up-regulated functions under 2MeSADP treatment, including previously described Dusp2 molecular activities such as phosphatase activity and phosphoprotein phosphatase activity. The complete list of genes in the up-regulated functional categories is provided as Supplementary file 1.

were among the possible candidates, including PTPs and DUSP families. In order to screen for a specific phosphatase that could explain the effect obtained with $P2Y_{13}$ agonist, we performed studies of gene expression by microarray analysis in granule neurons treated with 2MeSADP for 2 h, the incubation period in which 2MeSADP was more potent at inducing p38 dephosphorylation and exhibiting neuroprotective properties.

Gene expression analysis did not show any significant differences in 2MeSADP stimulated cells concerning up- or down-regulation of specific genes when they were analyzed individually. However, functional analysis revealed over-represented clusters of genes associated with specific functions that were significantly increased upon treatment with the nucleotide agonist. Over-represented GO terms related to Molecular Function and Biological Processes associated to protein phosphatase activity in 2MeSADP stimulated cells were: Protein dephosphorylation (GO:0006470), cellular protein modification process (GO:0006464), dephosphorylation (GO:0016311), post-translational protein modification (GO:0043687), phosphate-containing compound metabolic process (GO:0006796), enzyme binding (GO:0019899), hydrolase activity acting on ester bonds (GO:0016788), phosphatase activity (GO:0016791), phosphoprotein phosphatase activity (GO:0004721), protein kinase binding (GO:0019901), and kinase binding (GO:0019901) (Table 1).

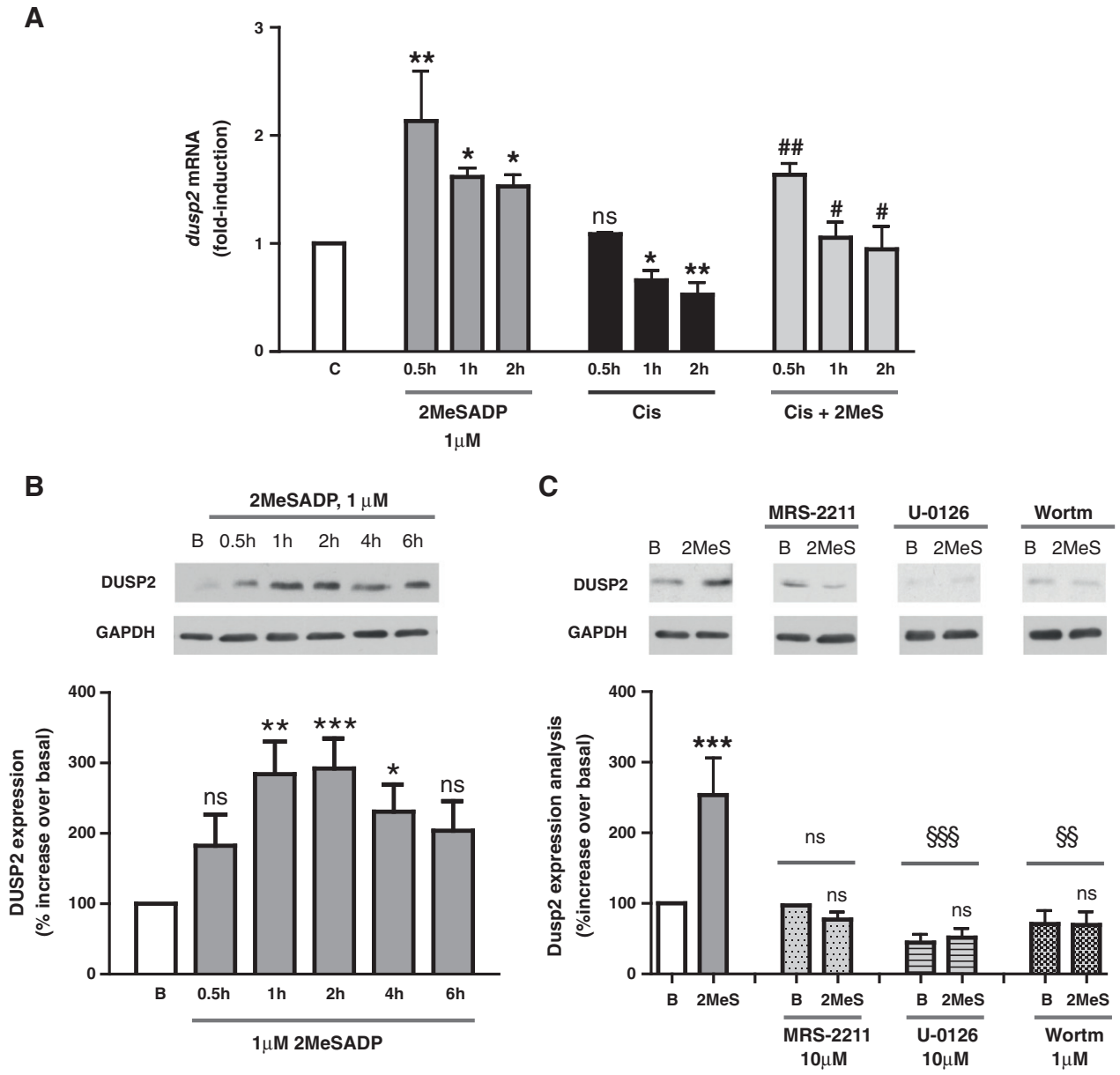

Fig. 5. $P2Y_{13}$ nucleotide agonist, 2MeSADP, induces the expression of DUSP2 in granule neurons. (A) Granule neurons were incubated with 1 μM 2MeSADP at different incubation periods and *dusp2* mRNA expression was analyzed by real-time Q-PCR as described in the Materials and methods. 20 μg/ml cisplatin was added alone or after 2 h incubation period with 1 μM 2MeSADP, and RNA collected at the indicated times. Values are expressed as fold-induction with respect to non-stimulated cells (Basal) after normalization with housekeeping GADPH levels. (B) and (C) DUSP2 protein levels were analyzed by immunoblot after the indicated incubation times with 1 μM 2MeSADP. In (C) pre-treatments were done for 10 min in the presence or absence of the $P2Y_{13}$ nucleotide antagonist, 10 μM MRS-2211, and for 30 min with the inhibitors, 10 μM U-0126 or 1 μM wortmannin, before the stimulation with 1 μM 2MeSADP for 2 h. The blots correspond to representative experiments. Histograms represent the percentage increase with respect to non-stimulated cells (100% basal), and were obtained by normalization of densitometric values with respect to GAPDH levels. Values are the means ± SD of experiments performed from different cultures (n = 5). Data were statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, when compared to basal (in the absence stimulation), and at §§$P < 0.01$, §§§$P < 0.001$, when treatment with inhibitors was compared to control conditions.

The full list of genes included in each one of these categories can be accessed through complete file enclosed in the Supplementary material.

The occurrence of several protein phosphatases in every list of grouped genes that belong to the family of dual specificity phosphatases, which were DUSP13, DUSP9 and DUSP2 was noteworthy. The last two are also termed "classical DUSPs" or MKPs, because they exhibit substrate specificity for MAP kinases. Meanwhile, DUSP13 is classified as "atypical DUSP", whose substrate and physiological function are still unknown [13]. Taking into account that DUSP13 was not related to MAPK function, it was dismissed for further analysis. Between classical DUSPs, DUSP9 belonged to the group of cytoplasmic constitutive phosphatases selective for ERK1/2, and it was excluded for our studies. Consequently, we focused our interest on DUSP2 inducible phosphatase, also termed as PAC-1, as a good candidate for mediating p38 dephosphorylation triggered by 2MeSADP in granule cells. It is to emphasize that this enzyme displays substrate selectivity for both p38 and ERK1/2 MAPKs, and exhibits nuclear localization [12].

3.6. DUSP2 protein phosphatase expression is induced by $P2Y_{13}$ receptor activation in granule neurons

Validation of data obtained in microarray analysis was undertaken by examining DUSP2 expression in cells stimulated with 2MeSADP nucleotide agonist. In accordance with the nature of *dusp2* as IEG, *dusp2* mRNA levels measured by real-time Q-PCR resulted to be increased 2-fold as early as 30 min incubation period with 2MeSADP, and remained elevated over basal until 1–2 h (Fig. 5A). As it can be observed in the figure, the exposure to the cytotoxic drug decreased *dusp2* mRNA levels in a time-dependent manner. This effect was counteracted when cells were pre-treated with the nucleotide agonist 2MeSADP (Fig. 5A).

Accordingly, DUSP2 expression at the protein level was also increased by stimulation with 2MeSADP, which was detectable as early as 30 min and reached maximum around 2 h (Fig. 5B). This was demonstrated to be a $P2Y_{13}$ receptor mediated effect, as was abolished upon treatment with the antagonist MRS-2211. In addition, 2MeSADP-induced DUSP2 expression was lost in the presence of the inhibitors, U-0126 and wortmannin, selective for MEK-1 (ERK1/2 upstream kinase) and PI3K, respectively (Fig. 5C). Therefore, $P2Y_{13}$ receptor activation was coupled to DUSP2 expression through the ERK1/2-canonical signaling described for this receptor in granule neurons, which was dependent on PI3K activity [20].

These data suggest that DUSP2 constitutes a specific target for $P2Y_{13}$ receptor activation, explaining its effects in restoring p38 MAPK signaling in granule neurons under conditions of genotoxic stress.

3.7. Analysis of DUSP2 protein phosphatase expression in granule neurons exposed to cisplatin and UV light

Subsequent studies were carried out to confirm the correlation between changes in DUSP2 activity and expression with altered p38 over-activation in granule neurons exposed to cisplatin and UV radiation. To address this goal, DUSP2 expression was analyzed in granule neurons after exposure to genotoxic stimuli. Cisplatin treatment induced a clear inverse relationship between DUSP2 protein levels and p38 phosphorylation over time (Fig. 6), which was in agreement with *dusp2* messenger transcriptional inhibition shown before (Fig. 5A). Cells exhibited low basal expression of DUSP2, as expected for an inducible protein phosphatase. However, treatment with cisplatin produced a progressive decrease in DUSP2 protein, inducing an almost complete loss after 16 h. This was coincident to maximal increases in phosphorylated p38 levels up to 8–10-fold within this incubation time. Interestingly, cisplatin used at a higher dose, 40 μg/ml, led to complete disappearance of DUSP2 protein beyond 30 min exposure period and to phosphorylated p38 signal saturation as soon as 2 h (Fig. 6). The same opposite pattern was observed between DUSP2 protein levels and phosphorylated p38 form after UV treatment, although in this case, DUSP2 expression loss

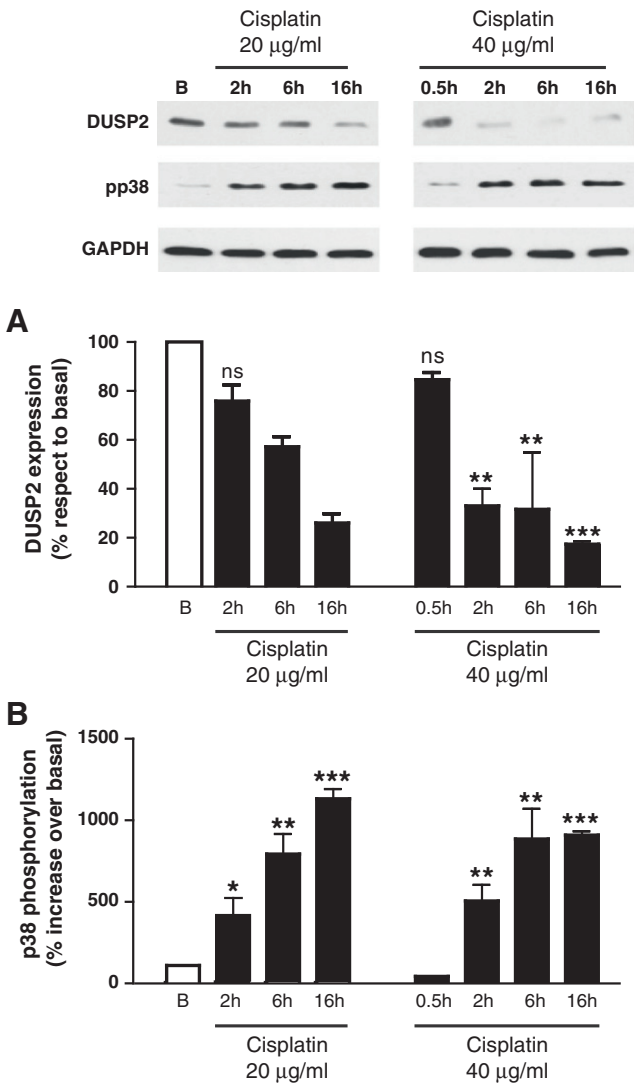

Fig. 6. Correlation between decrease of DUSP2 protein phosphatase expression and p38 sustained phosphorylation induced by cisplatin. Granule neurons were treated with cisplatin at two concentrations (20 and 40 μg/ml). Cell lysates were collected at different incubation periods to analyze DUSP2 protein phosphatase levels (A) and the phosphorylated form of p38 (B). The blots correspond to representative experiments. Histograms represent the percentage increase with respect to non-stimulated cells (100% basal), and were obtained by normalization of densitometric values with respect to GAPDH levels. Values are the means ± SD of experiments performed from different cultures (n = 4). Data were statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, when the effects were compared to basal (open bars, in the absence stimulation).

was observed at earlier incubation times of 2 h (Suppl. Fig. 2). These results indicate that impairment in DUSP2 protein phosphatase expression could be responsible of p38 MAPK signaling deregulation in granule neurons exposed to genotoxic stress.

3.8. Neuroprotection elicited by $P2Y_{13}$ receptor activation against cisplatin and UV-induced cell death

Granule cell viability was analyzed in conditions of genotoxic stress induced by cisplatin and UV light. As described in previous sections, caspase-3 dependent apoptotic pathway was activated after exposure to cisplatin (Fig. 1). This corresponded to decrease in cell viability close to 60% as quantified by the MTT survival assay. Previous stimulation with 1 μM 2MeSADP for 2 h significantly prevented the cytotoxic effect of the drug maintaining the cell survival up to 80%. 2MeSADP-mediated neuroprotective effect was completely lost when cells were

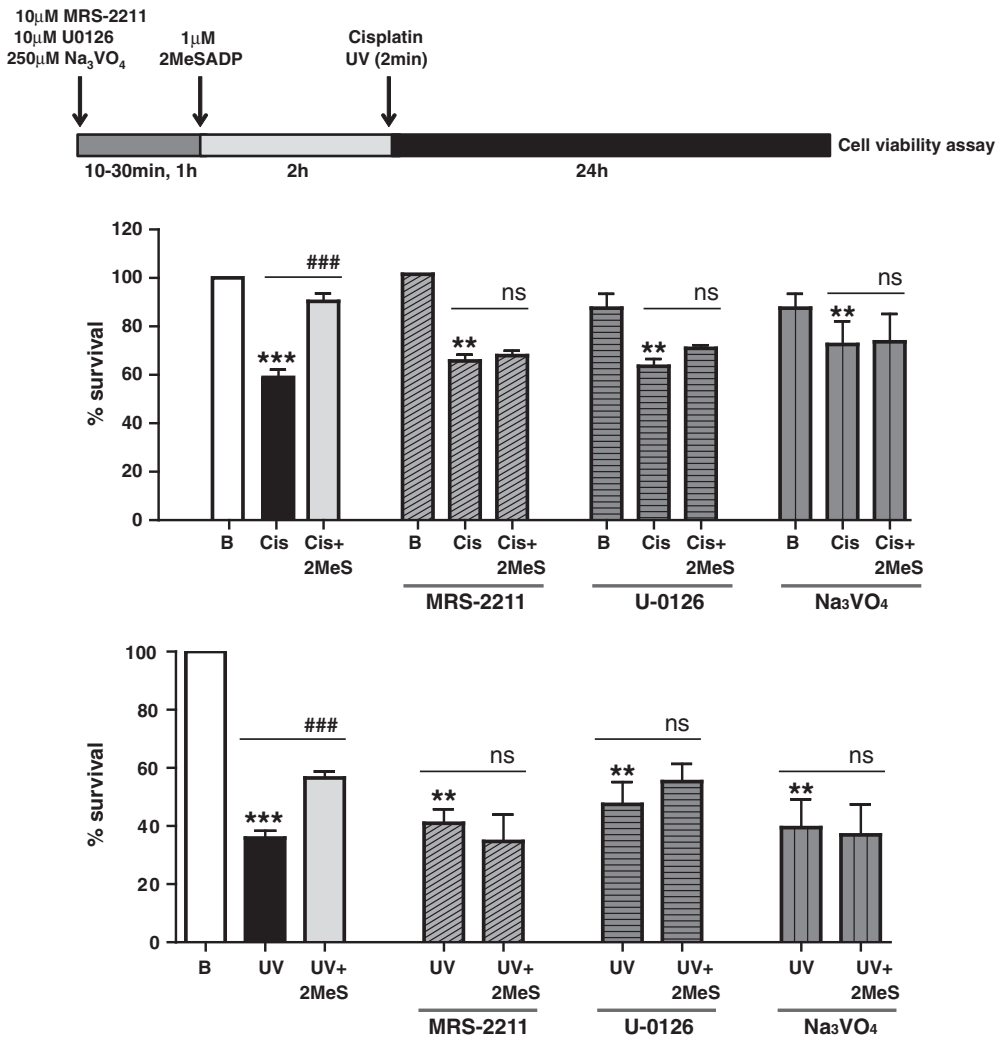

Fig. 7. $P2Y_{13}$ nucleotide agonist, 2MeSADP, rescues granule neurons from cisplatin- and UV-induced cell death. Granule neurons were submitted to different treatments previous to the addition of the nucleotide agonist 1 μM 2MeSADP: 10 min pre-incubation with the $P2Y_{13}$ antagonist, 10 μM MRS-2211, and 30 min or 1 h with the inhibitors, 10 μM U-0126 and 250 μM orthovanadate, respectively. Cytotoxic treatment was applied after 2 h stimulation with 2MeSADP, by the addition of 20 μg/ml cisplatin or the exposition to UV light for 2 min. Cell viability was measured 24 h later by the MTT assay, as described in the Materials and methods. Histograms represent the percentage of cell survival as compared to basal (100% survival, open bars, without cytotoxic treatment). Values are the means ± SD of experiments performed from different cultures (n = 10). Data were statistically significant at $^{**}P < 0.01$, $^{***}P < 0.001$, when the effects of cisplatin and UV light were compared to basal (open bars, in the absence stimulation), and at $^{###}P < 0.001$ or ns (non-significant), when each cytotoxic treatment was compared in the absence or presence of 2MeSADP.

pre-treated with the $P2Y_{13}$ antagonist, MRS-2211, and the ERK1/2 signaling transducing inhibitor, U-0126. In addition, when the protein tyrosine phosphatase inhibitor, orthovanadate, was tested, cell survival was severely compromised and 2MeSADP was no longer able to protect cells from death (Fig. 7). Similar results were observed when cells were exposed to UV radiation (Fig. 7). Although this treatment induced major levels of cell death, 2MeSADP was still able to maintain cell survival, with the same profile observed with cisplatin. Finally, as expected for P2Y13 mediated signaling, the use of the specific inhibitor of p38, SB202190, had no significant effect on the protective effect elicited by 2MeSADP. In addition, it did not protect granule neurons from toxicity induced by cisplatin and UV light (Supplementary Fig. 3). These data give evidence that 2MeSADP survival promoting effect was dependent on ERK1/2 signaling and that intact protein tyrosine phosphatase activity contributes to granule cell survival.

4. Discussion

The present study describes that $P2Y_{13}$ nucleotide receptor participates in the negative feedback regulation of p38 signaling in granule neurons. This effect is carried out through the activation of DUSP2 phosphatase expression, contributing to restoration of MAPK signaling, impaired upon conditions of genotoxic stress. This is the first study demonstrating coupling of nucleotide receptor to the expression of dual-specificity protein phosphatases in a neuronal cell model. In addition, this work emphasizes the relevance of protein phosphatase activity in the maintenance of both basal MAPK signaling and cell survival. First, it is demonstrated that p38 MAPK de-regulation induced by exposure to cisplatin and UV radiation is the consequence of transcriptional inhibition at the level of *dusp2* gene. Second, $P2Y_{13}$ receptor-induced neuroprotective effect resulted to be dependent on the recovery of main levels of phosphatase activity. Both, DUSP2 expression and neuroprotection, required PI3K/ERK1/2-dependent signaling in cerebellar granule neurons [20] (a simplified scheme is represented in Fig. 8).

Our data represent the first evidence of dual-specificity phosphatase regulation in neuronal cells through extracellular signals different from growth factors. Indeed, 2MeSADP nucleotide agonist acting on $P2Y_{13}$ receptor, increased *dusp2* mRNA levels that were early detected. This is consistent with *dusp2* being an immediately early inducible gene (IEG) and belonging to the group of nuclear inducible phosphatases

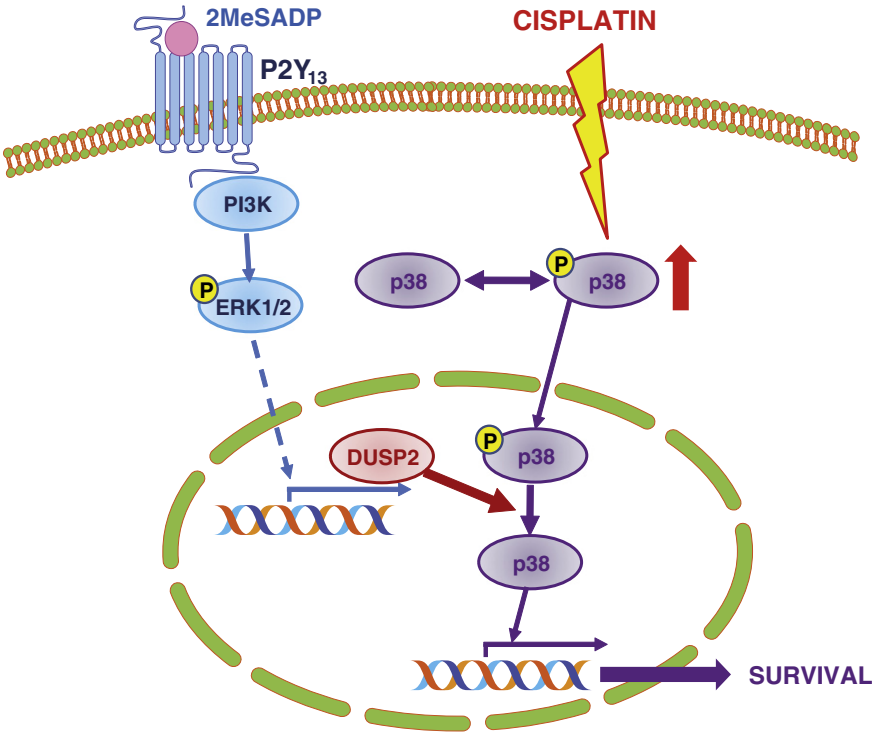

Fig. 8. $P2Y_{13}$ nucleotide receptor participates in the homeostasis of p38 signaling in granule neurons. Impaired inactivation of MAPK signaling is one of the consequences of DNA damage-induced neurotoxicity by cisplatin and UV radiation in granule neurons. Accumulation of p38 activated form can be identified as a response to transcriptional disruption of DUSP2 phosphatase elicited by these genotoxic agents. 2MeSADP nucleotide agonist acting through $P2Y_{13}$ receptor counteracts cisplatin and UV effects, by inducing the expression of *dusp2* to deactivate p38 in an ERK1/2-dependent manner, thereby providing a negative feedback loop to terminate p38 signaling. In addition, 2MeSADP also contributes to rescue granule neurons from cell death caused by genotoxic stimuli.

[12]. Accordingly, DUSP2 protein levels reached the maximal expression within a time window that is coincident with major effects observed for 2MeSADP at both p38 dephosphorylation and cell survival against cisplatin treatment. Therefore, both actions of the nucleotide agonist agree well with recovery of regular DUSP2 activity. It is necessary to highlight that ERK signaling is required for DUSP2 expression regulation elicited by $P2Y_{13}$ receptors, as previously described [32]. Indeed, DUSP phosphatase expression is usually under the control of MAPK activity [13,33]. This fact is reported for DUSP1 and DUSP6 submitted to the regulation of p38 and ERK1/2-dependent transcription factors, respectively [14,34]. Other kind of mechanisms, however, have been described in tumoral tissues, in which *dusp2* was a product of p53 protein transcriptional activation that would mediate the initiation of the apoptotic effect and the suppression of tumorigenesis [35].

It is worth mentioning that the impairment of DUSP2 correlates well with p38 sustained activation in granule neurons. Therefore, its substrate selectivity towards p38 MAPK protein [12,13] makes DUSP2 a good candidate for the maintenance of p38 signaling in this cellular model. In addition, ERK1/2 proteins also resulted to a long-term activation in response to genotoxic stimuli, in a similar way to that reported for other kinds of toxic conditions, such as glutamate excitotoxicity and trophic support deprivation in granule cells [36–38]. As deduced from the microarray gene expression data it cannot be discarded that other types of protein phosphatases, such as the ERK1/2-specific DUSP9 or PTPs, could be also relevant for the regulation of MAPK signaling in granule neurons stimulated with $P2Y_{13}$ agonists. Along this line, $P2Y_1$ nucleotide receptor was reported to regulate MAP kinase signaling through the activation of PTPs, protein tyrosine phosphatases, in cortical astrocytes. In this model, $P2Y_1$ receptor activation was protective against oxidative stress triggered by H_2O_2 treatment, through regulation of PTP activity and promoting the reestablishment of basal ERK1/2 signaling [39,40]. On the other hand, JNKs seemed not to play a relevant role in response to genotoxic stress in granule neurons. This could be explained on the basis that JNK basal activity is usually elevated in this model, and no further enhancement was obtained under these stress conditions. However, in tumoral cell lines, JNKs are strongly activated after UV exposure[41].

In relation to the outcome of long-term activation of MAPK after genotoxic stress, it seemed not to be associated to cell death in our experimental conditions. Cell viability experiments showed that neither ERK1/2 nor p38 inhibition was able to prevent cell death induced by cisplatin or UV (Fig. 7 and Supplementary Fig. 3). This contrasted to that described in other cell types in which apoptosis followed ERK1/2 or p38 sustained activation. In the case of granule neurons, it appears that other additional factors might contribute to apoptotic effect, as the nuclear accumulation of ERK1/2 or the activation of ROS [36]. In addition, other works supported that it was the concomitant activation of p38 and JNK together with the accumulation of JNK-target c-Jun, which was required for granule cell death [42,43].

The key role played by DUSP activity as one of the main mechanisms contributing to MAPK homeostasis is clarified by the data reported here. In fact, signaling termination processes are essential to ensure recovery of basal state, which allow cells to respond to ulterior stimuli and elicit proper physiological responses. As a significant point of control, deficiency in DUSP phosphatase activity appears to highly contribute to de-regulation of MAP kinase signaling that follows the onset of toxic or harmful stimuli [44]. Failure in this function can be accomplished by several mechanisms, such as degradation and transcriptional inhibition [45]. The later seems to be the mechanism contributing to DUSP2 deficiency in tumoral cells exposed to hypoxia and in inflammatory stress conditions [46,47]. Accordingly, a great deal of evidence suggests that recovery of DUSP activity is essential for cell survival. Indeed, DUSP1 and DUSP4 overexpression is enough to protect against cell death induced by UV light and cisplatin exposure in fibroblasts and different cell lines [34,41]. As an example in neural models, DUSP6 expression was able to rescue cortical neurons from cell death against toxic

glutamate [48] and the same is reported for DUSP1 that maintained cell viability versus NGF withdrawal in sympathetic neurons [49]. These data reported for the afore mentioned DUSPs support the results obtained in this study, in which restoration of DUSP2 expression contributes to the survival promoting effect of $P2Y_{13}$ nucleotide agonist, 2MeSADP, in granule neurons against genotoxic stimuli. In this regard, it should be emphasized that other factors besides DUSP2 regulation could be also participating in 2MeSADP-induced neuroprotection, which are inherent to $P2Y_{13}$ receptor coupled signaling. As we demonstrated in previous work, ERK-dependent CREB activation elicited by $P2Y_{13}$ receptors prevented glutamate-induced apoptosis [20]. In addition, $P2Y_{13}$ activation was able to rescue granule neurons from oxidative stress injury through the activation of the Nrf-2/heme oxigenase-1 axis, which was dependent on $P2Y_{13}$-mediated GSK3 signaling [21,30].

In conclusion, the present study contributes to demonstrate that nucleotides are relevant players in negative feedback regulatory mechanisms that result critical to maintain MAP kinase signaling homeostasis and cell survival. These processes can be then novel targets to be taken into consideration in conditions related to aging and neurodegenerative states, in which a great deal of signaling mechanisms result to be deregulated, MAPK signaling among them. The relevance of neuroprotective role played by nucleotides against cisplatin cytotoxicity is based on the use of this compound in chemotherapy treatments of neuroblastoma and other brain tumors, with great limiting side effects related to neurotoxicity, ototoxicity and peripheral neuropathy [50]. In addition cisplatin has been reported to induce defects in differentiation and synaptogenesis events during early development in hippocampal and cerebellar cell populations [51,52]. Overall these data demonstrates the pivotal role of $P2Y_{13}$ to cope with different types of damaging stimuli that compromise neural cell viability.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.05.004.

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