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P2 Receptor Antagonist Trinitrophenyl-Adenosine-Triphosphate Protects Hippocampus from Oxygen and Glucose Deprivation Cell Death

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ABSTRACT

In this work, we mainly used the organotypic model of rat hippocampus to demonstrate the protective role of the P2 receptor antagonist trinitrophenyl-adenosine-triphosphate (TNP-ATP) during oxygen/glucose deprivation. Among the P2X receptors that TNP-ATP specifically blocks, mainly $P2X_1$ seems to be involved in the processes of cell damage after oxygen/glucose deprivation. $P2X_1$ receptor is strongly and transiently up-regulated in 24 h after an ischemic insult on structures likely corresponding to mossy fibers and Schaffer collaterals of CA1–3 and dentate gyrus. Furthermore, $P2X_1$ receptor is down-regulated by pharmacological treatment with TNP-ATP, which is also found neuroprotective against ischemic cell death. Morphological studies conducted through immunofluorescence and confocal analysis in primary organotypic, in dissociated cultures, and in adult rat in vivo demonstrated the neuronal colocalization of $P2X_1$ protein with neurofilament light chain and neuronal nuclei immunoreactivity in myelinated and unmyelinated fibers of both granular and pyramidal neurons. In conclusion, with this work, we proved the neuronal distribution of $P2X_1$ receptor in hippocampus, and we presented evidence for a potential disadvantageous role of its expression during the path of in vitro ischemia.

Cerebral ischemia is one of the most common causes of death in aged people, being responsible for 10 to 12% of the world deaths per year (Hachinski, 2002). Ischemic injury involves a pronounced reduction in intracellular oxygen and glucose, which leads to rapid cell death associated with Ca^{2+}, Na^+, K^+, and Cl^- deregulation. In particular, the increase of Ca^{2+} influx can directly control the activation of proteolytic enzymes and apoptotic genes and the production of reactive oxygen species (Dirnagl et al., 1999). One class of receptors that directly controls the ionic calcium fluxes is the purinergic receptors (P2r) (Volonté et al., 2003; Franke and Illes, 2006) for extracellular ATP, a well known neurotransmitter and neuromodulator in the central nervous system (CNS) and peripheral nervous system (PNS) of adult mammals (Burnstock, 1990; Illes and Ribeiro, 2004; Fields and Burnstock, 2006), which mediates physiopathological functions ranging from proliferation, differentiation, and senescence to death (Volonté and Merlo, 1996; Volonté et al., 1999). P2r are divided in two subfamilies, ionotropic P2X and metabotropic P2Y receptors, which are distinct by molecular, pharmacological, and functional properties (Khakh et al., 2001; Gever et al., 2006; Volonté et al., 2006). Metabotropic P2Y are purine and pyrimidine receptors classified in eight different subtypes ($P2Y_{1,2,4,6,11–14}$), whose activation displays slow excitatory responses, phospholipase C, and 1,4,5-trisphosphate activation leading to cytosolic Ca^{2+} increase and cAMP modulation (Roberts et al., 2006). Ionotropic P2X receptors are instead characterized by fast excitatory neurotransmission, and they are divided into seven different subtypes ($P2X_{1–7}$) (North, 2002). Although each P2X receptor is defined by a specific nucleotide sequence, common to all the different subtypes is a direct influx of extracellular Ca^{2+} through the receptor channel. This leads to membrane depolarization, and consequently, to secondary activation of voltage-gated Ca^{2+} channels.

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ABBREVIATIONS: P2r, purinergic receptor(s); CNS, central nervous system; PNS, peripheral nervous system; OGD, oxygen/glucose deprivation; TNP-ATP, trinitrophenyl-adenosine-triphosphate; PBS, phosphate-buffered saline; NFL, neurofilament light; GFM, glucose-free medium; Ib_4, isolectin b4; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; PI, propidium iodide; DG, dentate gyrus; mf, mossy fibers; sc, Schaffer collateral fibers; NeuN, neuronal nuclei.

Both P2X and P2Y receptors are ubiquitously expressed in the CNS and PNS (Kucher and Neary, 2005). In particular, the $P2X_1$ receptor is present on astrocytes of juvenile rats (Kukley et al., 2001), on rat cerebellar granule neurons (Amadio et al., 2002), and on purified synaptosome from rat hippocampus (Rodrigues et al., 2005). Although the function of the $P2X_1$ receptor is poorly described in the CNS (Brown et al., 2002; Aschrafi et al., 2004), more information is available for the PNS, where this receptor apparently participates to activation of pathological pain states (Chizh and Illes, 2001). Moreover, a role for various P2 receptors in the path of oxygen/glucose deprivation (OGD) is well established. In particular, we showed previously the presence and activation by OGD of microglial $P2X_4$ in organotypic hippocampal slices (Cavaliere et al., 2003) and of neuronal $P2X_2$ and $P2X_7$ receptors (Cavaliere et al., 2002, 2004b; Melani et al., 2006); and in human neuroblastoma cells, we showed that heterologous expression of metabotropic $P2Y_4$ receptor exacerbates cell death induced by metabolic impairment (Cavaliere et al., 2004a). Consistently, neurodegeneration induced by OGD is prevented by several different P2r antagonists (Cavaliere et al., 2001, 2003, 2004a,b, 2005).

In this work, we thus investigated the biological effect of the P2 receptor antagonist trinitrophenyl-adenosine-triphosphate (TNP-ATP) particularly during OGD and the potential role of the $P2X_1$ receptor in this detrimental process in rat hippocampus. We found a direct time- and dose-dependent involvement of this receptor in the path of OGD-induced cell death.

Materials and Methods

Cell Culture

Hippocampal Organotypic Cultures. Slice cultures were prepared using a modification of the method by Stoppini et al. (1991). In brief, Wistar rat pups (8–10 days old) were sacrificed, and their brains were removed. Hippocampi were excised, cut at 400 μm in thickness on a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, UK), and separated into Hanks' balanced salt solution (0.185 mg/ml $CaCl_2$, 0.1 mg/ml $MgSO_4$, 0.4 mg/ml KCl, 0.06 mg/ml KH_2PO_4, 8 mg/ml NaCl, 0.05 mg/ml Na_2HPO_4, 0.35 mg/ml $NaHCO_3$, and 1 mg/ml glucose). Slices were plated on Millicell CM inserts (Millipore, Milan, Italy), and plates were maintained in 75% HME 03 (Cell Concept, Umkirch, Germany) and 25% horse serum (Invitrogen, Milan, Italy) for 3 days at 37°C, and then they were shifted to 33°C in Neurobasal medium supplemented with 0.5% B27 (both from Invitrogen). Experiments were performed after 12 to 14 days in vitro.

Primary Dissociated Hippocampal Cultures. Primary dissociated hippocampal cells were prepared from P2 rat pups. In brief, hippocampi from both hemispheres were dissected into small tissue blocks. Slices were then incubated in papain solution (116 mM NaCl, 5.4 mM KCl, 26 mM $NaHCO_3$, 1 mM $NaHPO_4$, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 mM EDTA, 25 mM glucose, and 200 U of papain 200) for 15 min, and then they were dissociated with a glass Pasteur pipette. Dissociated cells were washed in Earl's balanced salt solution and plated in Eagle's minimal essential medium, 10% dialyzed fetal bovine serum with 0.5 mM glutamine, 20 mM glucose, and gentamicin (all from Invitrogen). The cell density at plating was approximately 100 to 200 cells/mm^2. Proliferation of non-neuronal cells was inhibited at 2 days after plating by the addition of 10 μM 1-γ-D-arabinofuranosyl cytosine (Sigma, Milan, Italy) to the growth medium. Experiments were performed after 8 to 10 days in vitro.

Histological Procedures

Adult Wistar rats weighing 200 to 250 g (Harlan, San Pietro al Natisone, Udine, Italy) were anesthetized by intraperitoneal injections of 60 mg/kg sodium pentobarbital, and they were perfused transcardially with 250 ml of saline at room temperature, followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, postfixed in the same fixative for 2 h, and then they were transferred to 30% sucrose in phosphate buffer at 4°C until they sank. In all preparations, animals were handled in accordance with the European Community Council Directive and in accordance with the Declaration of Helsinki. All possible efforts were made to minimize animal suffering and number of animals used.

Immunofluorescence and Confocal Analysis

Organotypic Slices. Treated slices were fixed for 40 min in 4% paraformaldehyde, and slices were saturated at room temperature in 10% donkey normal serum (Alomone Labs, Jerusalem, Israel) in PBS. The slices were then incubated overnight at 4°C with different primary antisera in 1% donkey normal serum in PBS [rabbit anti-$P2X_{1(382–399)}$ from Alomone Labs was used at 1:500; goat anti-neurofilament light chain (NFL) (C15) from Santa Cruz Biotechnology, Inc. (Milan, Italy) was used at 1:100; goat anti-doublecortin (C18) (Santa Cruz Biotechnology) was used at 1:200; mouse anti-βIII tubulin from Promega (Milan, Italy) was used at 1:2000; biotinylated isolectin b4 (Ib_4) from *Griffonia simplicifolia* seeds from Sigma was used at 10 μg/ml; mouse antiglial fibrillary acidic protein (GFAP) (R&D, Milan, Italy) was used at 1:100; and mouse antimyelin basic protein (MBP) from Roche Diagnostics (Monza, Italy) was used at 1:200]. After further washing, the cultures were incubated in a solution containing a mixture of secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA; Cy2-conjugated donkey anti-rabbit IgG and/or Cy3-conjugated donkey anti-mouse and anti-goat IgG; all used at 1:100). After further washing, the plates were coverslipped with Gel/Mount anti-fading mounting medium (Biomeda, Foster City, CA).

Primary Dissociated Cultures. Cultures were fixed for 10 min in 4% paraformaldehyde and permeabilized for 5 min in 0.1% Triton X-100 in PBS. Nonspecific sites were saturated in 10% donkey normal serum for 30 min at room temperature. Anti-$P2X_1$ and anti-NFL primary antibodies were used at 1:1000 and 1:300, respectively, for 90 min at room temperature. Cells were washed three times, and then they were incubated with secondary antibodies (Cy2-conjugated donkey anti-rabbit IgG and/or Cy3-conjugated donkey anti-mouse IgG; all at 1:200). After further washes, the cells were coverslipped, and then they were analyzed using an LSM 510 scanning confocal microscope (Carl Zeiss, Milan, Italy), equipped with an argon laser emitting at 488 nm and a helium/neon laser emitting at 543 nm

Slices from Adult Rat Hippocampus. Transverse sections 40 μm in thickness were cut on a freezing microtome (Leitz, Oberkochen, Germany), and then they were processed for double-immunofluorescence studies. Nonspecific binding sites were blocked with 10% normal donkey serum in 0.3% Triton X-100 in PBS for 30 min at room temperature. The sections were incubated in a mixture of primary antisera for 24 h in 0.3% Triton X-100 in PBS. Rabbit anti-$P2X_1$ (1:300) was used in combination with goat anti-NFL (1:100) or with mouse anti-MBP (1:200). The secondary antibodies used for double labeling were Cy3-conjugated donkey anti-rabbit IgG (1:100; red immunofluorescence) or Cy2-conjugated donkey anti-goat IgG (1:100; green immunofluorescence) or Cy2-conjugated donkey anti-mouse IgG (1:100; green immunofluorescence). The sections were washed in PBS three times for 5 min each, and then they were incubated for 3 h in a solution containing a mixture of the secondary antibodies in 1% normal donkey serum in PBS. After further washes, the sections were mounted on slide glasses and allowed to air dry. Slides were then coverslipped with Gel/Mount anti-fading mounting medium.

All P2r antibodies were affinity-purified and raised against highly

purified peptides (identity confirmed by mass spectrometry and amino acid analysis), corresponding to epitopes specific for each P2 receptor subtype and not present in any other known protein. The specificity of the labeling was confirmed by incubations performed with the secondary antibodies in the absence of the primary antisera and also by incubations with the primary antisera in the presence of the immunogenic neutralizing peptide. No immunoreactivity was observed under these conditions. Immunofluorescence was visualized by an LSM 510 scanning confocal microscope (Carl Zeiss).

Oxygen/Glucose Deprivation

Glucose-free medium (GFM; 120 mM NaCl, 4 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 2 mM KH_2PO_4, and 2 mg/ml mannitol, pH 7.4) was saturated with N_2. The inserts with organotypic slices were placed in 1 ml of saturated GFM, and they were maintained at 37°C for 40 min in an N_2-saturated hypoxic chamber (Billups-Rothenberg, Del Mar, CA). In the control conditions, medium consisted of GFM supplemented with 1 mg/ml glucose instead of mannitol. The GFM was replaced with the Neurobasal medium, and the cultures were kept under normoxic conditions for additional 24 h at 33°C.

Total Protein Extraction

Organotypic cultures were maintained under OGD conditions in the simultaneous presence or absence of 50 μM TNP-ATP (Sigma). After 24 h, four organotypic slices from each experimental condition were extracted in radioimmunoprecipitation assay buffer (PBS supplemented with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 μM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin; all from Sigma), and then homogenized. They were maintained for 1 h on ice, sonicated, and centrifuged at 4°C at 10,000*g* for 10 min. Protein quantification was performed in the supernatants by Bradford colorimetric assay (Bio-Rad, Segrate, Milan, Italy).

Western Blot

An equal amount of total protein from each sample (50 μg) was separated by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel, and then protein was transferred overnight onto a nitrocellulose membrane (Hybond C; GE Healthcare, Cologno Monzese, Milan, Italy). The filters were prewetted in 5% nonfat milk in 10 mM Tris, pH 8, 150 mM NaCl, and 0.1% Tween 20, and then they were hybridized for 3 h with anti-$P2X_1$ antisera (1:200). All antisera were immunodetected with an anti-rabbit horseradish peroxidase-conjugated antibody and developed by enhanced chemiluminescence (Santa Cruz Biotechnology, Inc.). Quantification of specific bands was performed in a linear range of detection, using Kodak 1D 3.5.3 software (Eastman Kodak, Rochester, NY).

Quantitative Analysis of Cell Death

Cell death in organotypic cultures was evaluated by uptake of propidium iodide (PI) (Sigma) as established by Pozzo Miller et al. (1994) and Chechneva et al. (2005). Slices were incubated for 2 h at 37°C with PI-containing medium at a final concentration of 10 μM. The fluorescent dye intercalates into DNA of cells that have lost membrane integrity; therefore, it represents a marker of cell death. Afterward, the slices were excited with a 510- to 560-nm light, and the emitted fluorescence was acquired at 610 nm, using a rhodamine filter on an inverted fluorescence microscope (Eclipse TE 300; Nikon, Duesseldorf, Germany). Images were taken using a charge-coupled device camera and analyzed with image analysis software (LUCIA; Nikon). Damage was given as percentage of the CA1–3 area labeled by PI-positive signal. Images were taken with 5× magnification, and panels were arranged with IAS 006 (Delta Sistemi, Milan, Italy).

Statistical Analysis

Values were normalized as specified in each figure legend. Statistical differences were evaluated by one-way analysis of variance, followed by post hoc test (Tukey's honestly significant difference). All values reported are significant, with at least $p < 0.05$.

Results

With the aim to test the pharmacological outcome of inhibiting especially $P2X_1$ receptor during an OGD insult, we cultured hippocampal organotypic slices at 10 to 12 days in vitro for 40 min in OGD and with or without the specific $P2X_1$ receptor antagonist TNP-ATP. We performed a dose-response experiment with a concentration range of 10, 50, and 200 μM (Fig. 1A, samples 1, 2, and 3, respectively). PI incorporation during OGD revealed cell damage or death in the CA1–3 neuronal layers, which was reduced by 30 to 40% and 70% in the presence of 50 and 200 μM TNP-ATP, respectively (Fig. 1, B and C). The lower concentration of 10 μM reduced cell death by only 10 to 15%. For further experiments, we thus used the concentration of 50 μM to distinguish between specificity and effectiveness. When we pretreated with TNP-ATP for 1 h before OGD (Fig. 1, sample 4), the entire hippocampal slice tissue, with the exception of the CA2 region, showed cell damage reduced 50% with respect to the control condition. Nevertheless, TNP-ATP was inefficient when

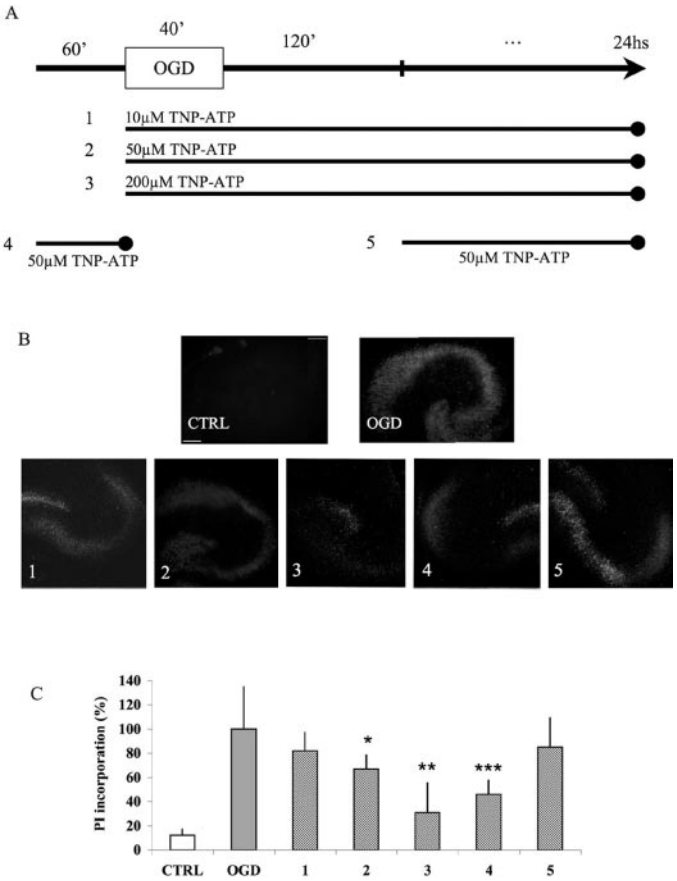

Fig. 1. The antagonist TNP-ATP reduces neuronal damage by OGD. Hippocampal slices at 12 days in vitro were subjected to OGD for 40 min. Control slices were maintained in 1 mg/ml glucose, whereas different concentrations of TNP-ATP were added during and after OGD (sample 1, 10 μM; sample 2, 50 μM; and sample 3, 200 μM). Treatment of slices with 50 μM TNP-ATP was also performed 1 h before OGD (sample 4) and 2 h after OGD (sample 5). Twenty-four hours after the insult, cell death was visualized by PI incorporation (B), and it was measured by densitometric analysis (C). Statistical differences were calculated versus OGD condition (100% PI incorporation) at least with $p < 0.05$.

given 2 h after OGD. The pharmacological role of TNP-ATP is generally associated to the antagonistic block of purinergic $P2X_1$ and $P2X_{2/3}$ receptors (Lewis et al., 1998; Rodrigues et al., 2005). Because we already described the involvement of neuronal $P2X_2$ in ischemic events in the hippocampus (Cavaliere et al., 2003), here we investigated the possible functional contribution of $P2X_1$ and $P2X_3$ receptors. Because we did not find evidence for $P2X_3$ receptor modulation after OGD (data not shown), we concentrated our studies on the expression of $P2X_1$ receptor on rat hippocampus, as analyzed by confocal immunofluorescence in organotypic slice cultures at 10 to 12 days in vitro. We found that $P2X_1$ receptor protein is abundantly distributed on fibers in neuronal layers CA1–3 and in dentate gyrus (DG), probably corresponding to mossy fibers (mf) and Schaffer collaterals (sc) as shown by morphological confocal analysis (Fig. 2). In different hippocampal regions, we demonstrated colocalization of $P2X_1$ protein with NFL both on dendritic fibers and around neuronal bodies (Fig. 3, A and B). Double immunofluorescence with NeuN confirmed the neuronal expression of $P2X_1$ receptor (Fig. 3C). Negative colocalization with the neuronal markers doublecortin and βIII tubulin, which are mainly expressed on neuronal precursors in the neurogenic region of dentate gyrus, instead indicated a lack of $P2X_1$ receptor expression in the early phases of neuronal maturation (data not shown). Immunofluorescence analysis of $P2X_1$ and NFL on primary dissociated hippocampal neurons confirmed the neuronal localization of $P2X_1$ protein on both pyramidal (Fig. 3D) and granular (Fig. 3E) phenotypes. Moreover, we excluded the concomitant presence of $P2X_1$ receptor on microglia, astrocytes, and oligodendrocytes, by lack of colocalization with the microglial marker Ib_4, the astrocytic GFAP, and the oligodendrocytic MBP (Fig. 4). The absence of colocalization with

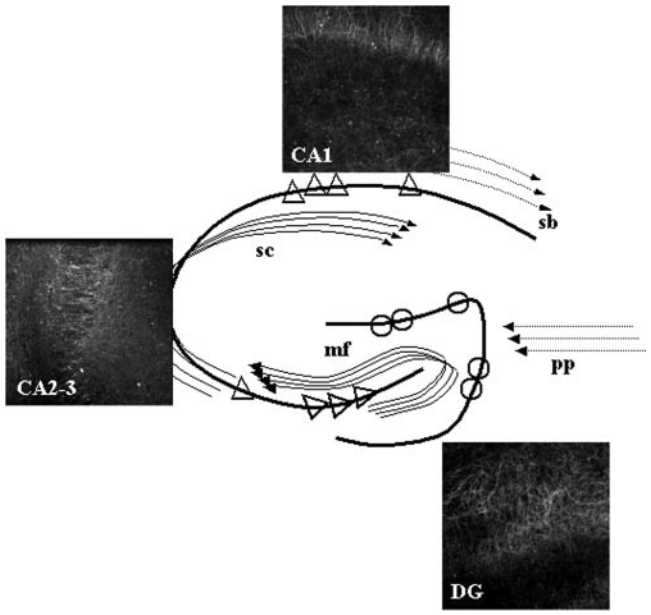

Fig. 2. $P2X_1$ receptor is expressed on fibers in CA1–3/DG hippocampal region. Hippocampal slices at 12 days in vitro were fixed and immunostained with anti-$P2X_1$ antiserum. Images were taken at 10× magnification on different regions (CA1–3 and DG). The scheme represents neuronal circuits of hippocampus. Pyramidal neurons on CA1 and CA3 are shown as triangles, and open circles represent granular neurons. Afferent fibers on perforant pathway from entorhinal cortex (pp) and deferent fibers from CA1 to subiculum (sb) are represented as dotted lines; mf from DG to CA3 and sc from CA3 to CA1 are represented as lines.

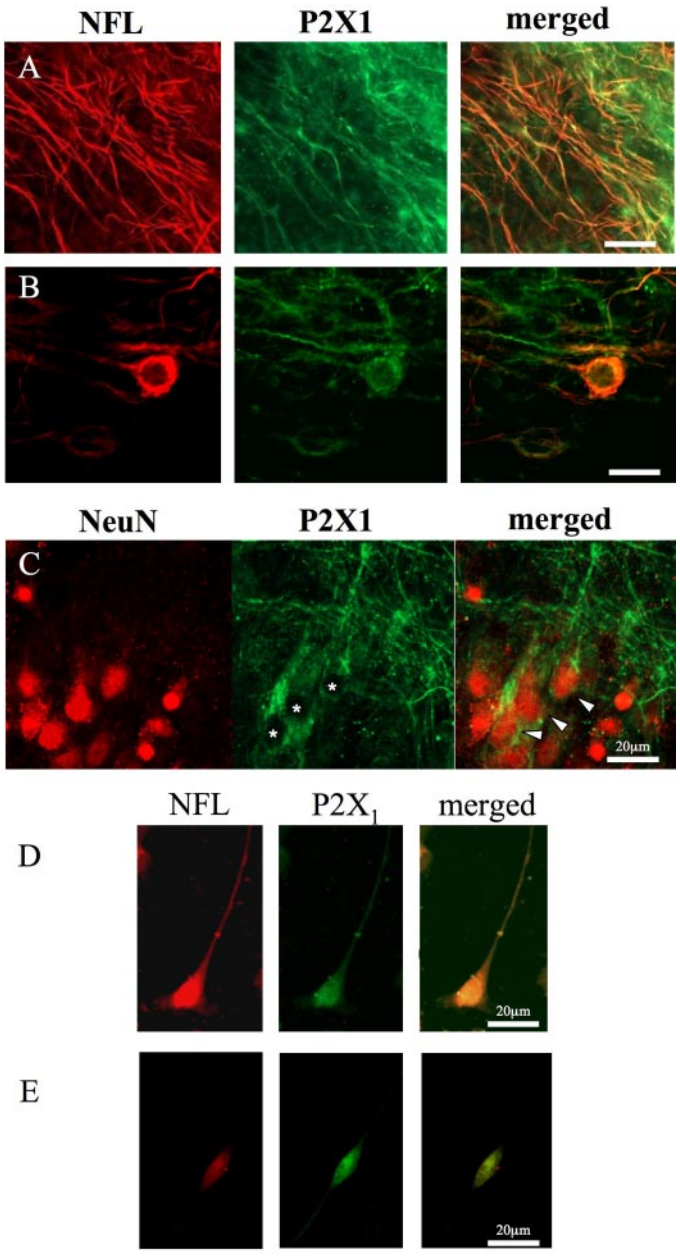

Fig. 3. $P2X_1$ receptor expression on neuronal fibers. Hippocampal slices at 12 days in vitro (A–C) and primary dissociated hippocampal neurons at 8 days in vitro (D and E) were fixed and double immunostained with anti-$P2X_1$ receptor antisera (green), NFL (A, B, D, and E), and NeuN (C), both red. Images were taken from dentate gyrus (A and B) and CA1 (C) regions. Neuronal bodies in C are marked with an asterisk, and arrows show the $P2X_1$-positive fibers. Colocalization of $P2X_1$ receptor with NFL was also demonstrated on pyramidal (D) and granular (E) dissociated neurons, as shown on merged pictures at 40× magnification.

MBP further suggested that $P2X_1$ receptor is localized on unmyelinated neuronal fibers, at least in hippocampal slices. We could not establish whether $P2X_1$ receptor was also present on myelinated afferent and deferent fibers, since the organotypic slice preparation per se removes both myelinated fibers coming from the entorhinal cortex and those projecting to the subiculum. For this reason, we then analyzed directly in situ the presence of $P2X_1$ receptor on hippocampal slices of adult rat. Colocalization with NFL was confirmed (Fig. 5A), and confocal double immunofluorescence of $P2X_1$ receptor with MBP in the hilus (the hippocampal region receiving

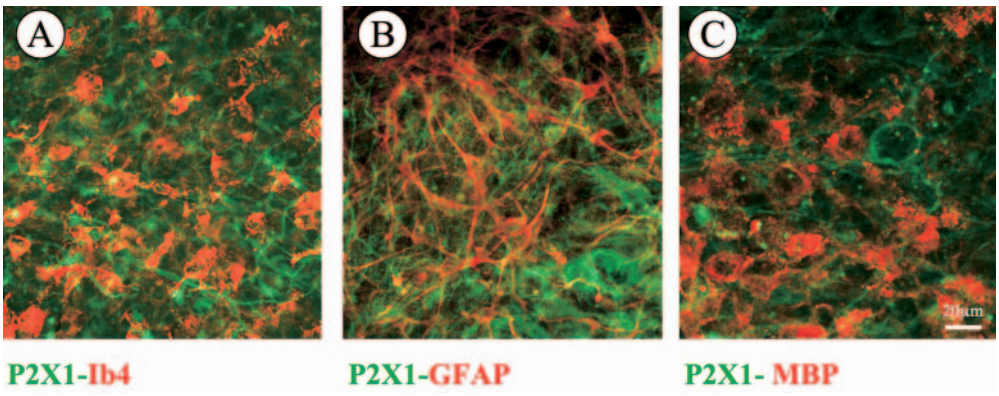

Fig. 4. $P2X_1$ receptor is not expressed on glia cells. Organotypic cultures at 12 days in vitro were fixed and double immunostained for $P2X_1$ receptor (green) and different glial markers (red) (A, Ib_4 for microglia; B, GFAP for astrocytes; and C, MBP for oligodendrocytes). Only merged panels on CA3/DG regions are represented.

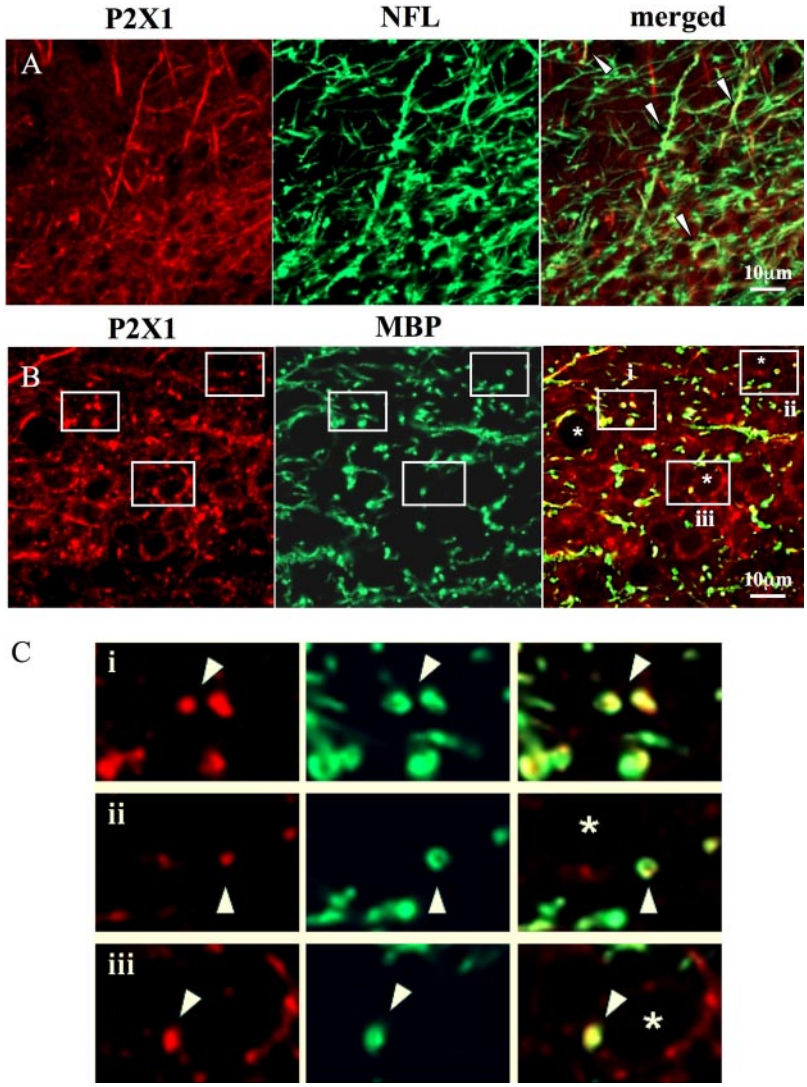

Fig. 5. $P2X_1$ receptor is present on myelinated and unmyelinated fibers of adult rat hippocampus. Slices from adult rat hippocampus were double immunostained for $P2X_1$ receptor (red) and NFL (A), or MBP (B), in the region of the hilus. The squares in B show myelinated afferent fibers (merged) transversally sectioned and adjacent to neuronal bodies (asterisks). In C, three examples of myelinated fibers (boxed i, ii, and iii) are shown at 40× magnification. Asterisks represent neuronal bodies. Arrows show the colocalization.

myelinated afferent fibers) showed that, in transversally sectioned myelinated fibers, MBP immunoreactivity partially overlapped with $P2X_1$ receptor signal (Fig. 5, B and C). The merged field conferred only apparent colocalization between $P2X_1$ and MBP, due to close location of the two immunoreactive structures. Double immunofluorescence of $P2X_1$ with microglial and astroglial markers (Ib_4 and GFAP, respectively) provided negative results also in adult hippocampal slices in vivo (data not shown).

Moreover, immunofluorescence confocal analysis revealed a significant increase in $P2X_1$ receptor expression occurring 24 h after OGD on fibers of the stratum oriens and radiatum and on fibers that cross the granular layer (Fig. 6, A and B). Pharmacological treatment with 50 μM TNP-ATP during and after OGD reduced $P2X_1$ receptor expression in all these regions. Different doses of TNP-ATP used during OGD demonstrated that the concentration-response curves for prevention of both cell death and modulation of $P2X_1$ receptor were

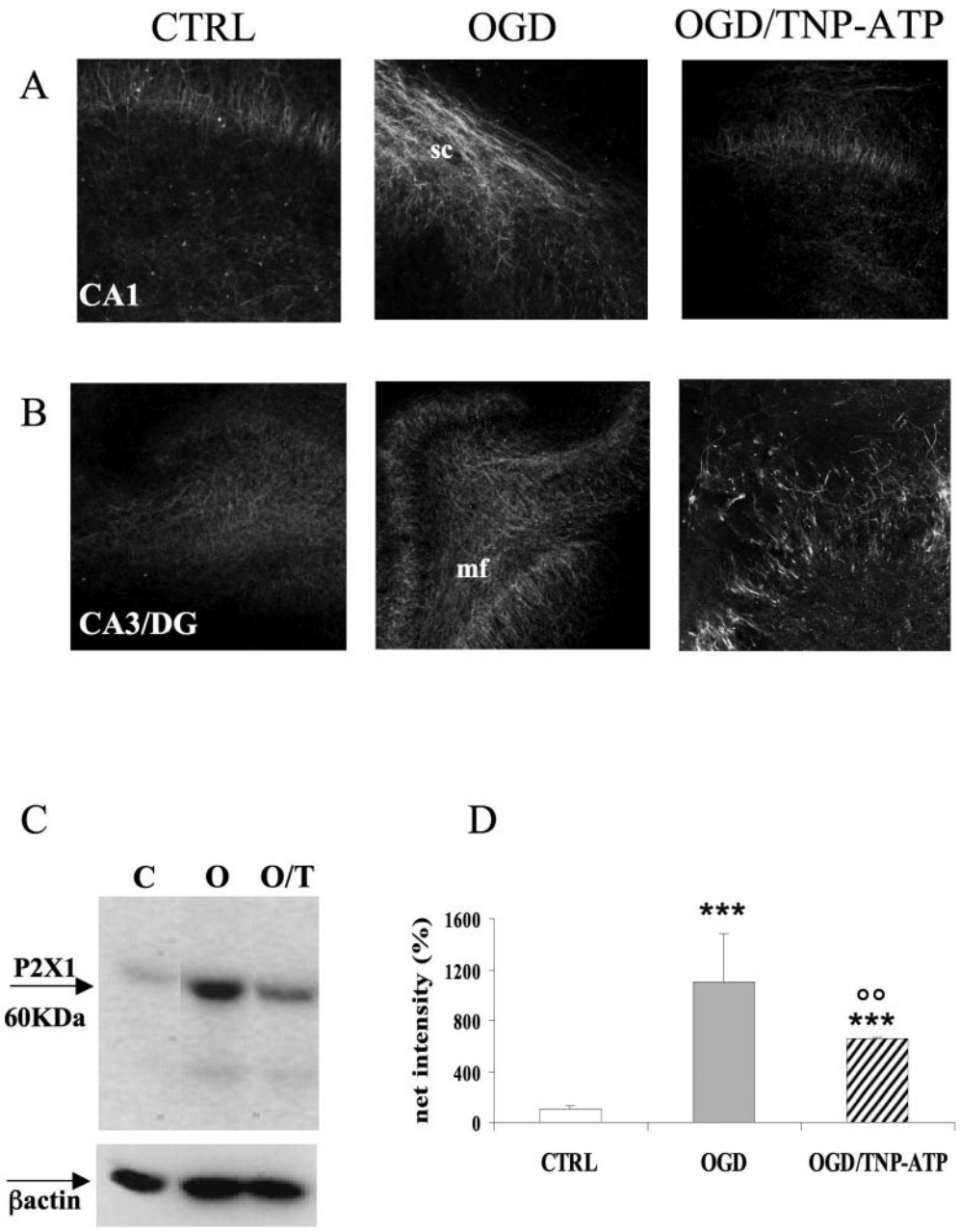

Fig. 6. P2X$_1$ receptor expression is modulated after OGD. Organotypic cultures at 12 days in vitro were subjected to OGD for 40 min in the absence or presence of 50 μM TNP-ATP. Immunofluorescence (A and B) and Western blot analysis (C) were performed 24 h later. P2X$_1$ receptor was detected on CA1 (A) and CA3/DG (B) with 20× magnification. After Western blot analysis (C), P2X$_1$ protein intensity was quantified by densitometric analysis, after normalization with β-actin signal (D). In C, C, 1 mg/ml glucose control; O, OGD; and O/T, OGD plus 50 μM TNP-ATP. Statistical differences shown in D were calculated versus control condition with $p < 0.001$ (stars) and versus OGD with $p < 0.05$ (open circles). The results are representative of at least four independent experiments.

very similar (data not shown). The results were confirmed by Western blot analysis showing that P2X$_1$ receptor expression was induced of approximately 10-fold with respect to control at 24 h after OGD, whereas TNP-ATP reduced P2X$_1$ protein expression by approximately 35% with respect to OGD (Fig. 6, C and D). We next measured the damage produced at 24 or 48 h after OGD. We found that cell damage or death visualized by PI gradually increased up to 48 h, whereas P2X$_1$ receptor protein was maximally induced at 24 h and it decreased at 48 h, therefore, preceding the maximal extent of cell death (Fig. 7, A and B).

Discussion

In this work, we described the expression of purinergic P2X$_1$ receptor on rat hippocampal postmitotic neurons, and we discussed its participation particularly in OGD-induced neuronal death. In contrast to P2X$_7$ receptor localized mainly at synaptic terminals (Cavaliere et al., 2004b) but similarly to P2X$_2$ receptor present on neurons (Cavaliere et al., 2003), P2X$_1$ protein is localized on neuronal fibers and cell bodies in both primary dissociated pyramidal and granular neurons in CA1–3 and DG layers of organotypic slices and also in adult rat hippocampus. Specificity of the P2X$_1$ receptor signal is proved by the result that although double immunofluorescence with NeuN confirms the neuronal expression of P2X$_1$ receptor on myelinated and unmyelinated fibers of CA1–3 and dentate gyrus, the P2X$_7$-positive signal also present in CA1–2 pyramidal cell layer throughout the strata oriens and radiatum colocalizes neither with NeuN nor with MBP (Cavaliere et al., 2004b). Thus, this difference in localization would indicate a different expression of these two receptor subtypes in the same hippocampus. Moreover, neither P2X$_1$ nor P2X$_2$ receptor signals overlap, in view of the fact that,

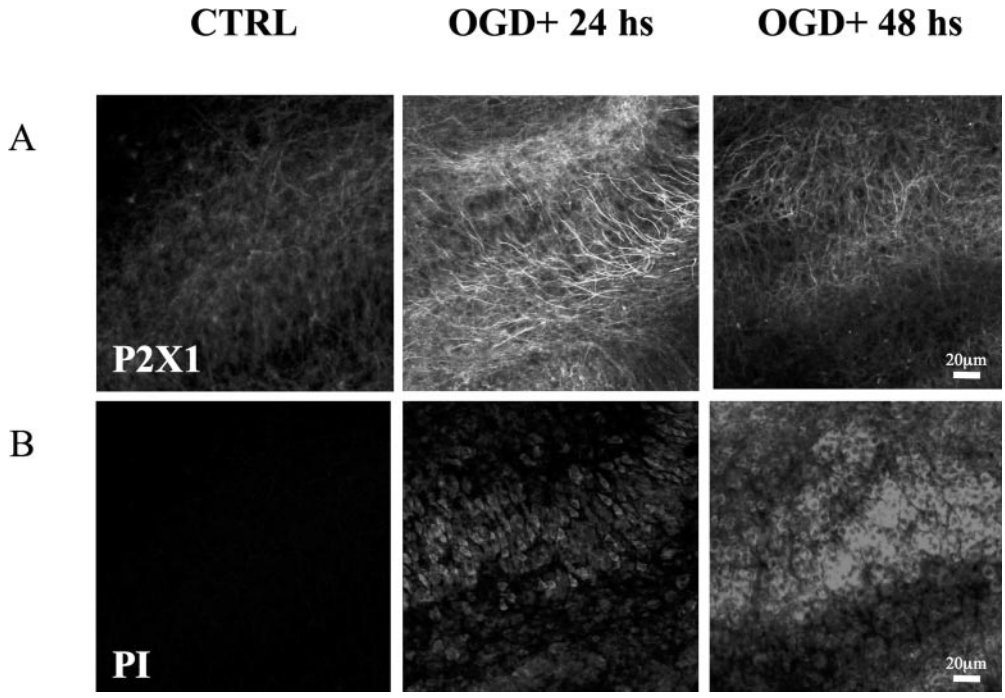

Fig. 7. $P2X_1$ receptor expression precedes in time the maximal extent of cell death. Organotypic cultures were subjected to OGD for 40 min, and they were subsequently stained with PI (B; red). Twenty-four and 48 h after the insult, $P2X_1$ expression (A; green) and cell damage (B; red) were evaluated by immunofluorescence and PI incorporation in CA3 hippocampal region.

under identical ischemic conditions (40 min of OGD followed by 20–24 h post-treatment), the two proteins elicit different levels of induction (10- and 2.5-fold, respectively; also see Cavaliere et al., 2003). Finally, the possibility of cross-reactivity of $P2X_1$ receptor is excluded with either $P2X_3$ subtype (not apparently modulated by ischemic conditions) or $P2X_4$ subtype (expressed on microglia cells and not on neurons; Cavaliere et al., 2003). All this evidence demonstrates the high degree of heterogeneity, and, simultaneously, the specificity of the cell and tissue distribution of different P2 receptor subtypes in the hippocampus, as plausible indications of a diverse functional role of these receptors in this specific brain area. Because of its topographic distribution and by the lack of colocalization with various glial markers in both organotypic cultures and adult rat hippocampus, we assumed that the $P2X_1$ receptor is expressed on mossy fibers and Schaffer collaterals. In particular, the absence of colocalization with MBP in organotypic cultures sustained the presence of $P2X_1$ receptor on unmyelinated fibers of the internal circuit, which are described in the hippocampal CA1–3 areas as thin axons with no myelin sheath (Shepherd and Harris, 1998). Nonetheless, studies performed on adult rat hippocampus demonstrated the simultaneous expression of $P2X_1$ receptor also on afferent myelinated fibers, suggesting that this receptor as a general marker for the entire hippocampal circuitry, independently from the granular versus pyramidal neuronal phenotypes, or from the different electrical conductance of myelinated versus unmyelinated fibers. In this regard, our work establishing the presence of $P2X_1$ receptor on neurons of CA1–3 and DG rat hippocampus extends previous results obtained for astrocytes of stratum oriens, radiatum, and lacunosum (Kukley et al., 2001).

The general mechanistic role played by purinergic receptors in cell loss induced by various noxious insults such as ATP, glutamate excitotoxicity, growth factor deprivation, and hypoglycemia and chemical hypoxia is well known (Volonté et al., 2003, 2006). For example, $P2X_4$ receptor is up-regulated in hippocampus on microglia cells during OGD-induced gliosis (Cavaliere et al., 2003); $P2X_7$ receptor is expressed, respectively, on neuronal synapses in hippocampus (Cavaliere et al., 2004a) or on microglia in cerebral cortex and striatum only after in vitro or in vivo ischemia (Melani et al., 2006); and $P2X_2$ present on neuronal fibers in hippocampus is directly involved in ischemic cell death (Cavaliere et al., 2003). We now strengthened and extended the finding of those studies to the $P2X_1$ receptor, proving that $P2X_1$ protein expression also is highly augmented in hippocampus by OGD, although exclusively on postmitotic mature neuronal cells, and that the selective ATP analog and nearly specific $P2X_1$ receptor antagonist TNP-ATP can reduce both ischemic damage and $P2X_1$ protein expression. The functional down-regulation of P2X receptors protein by antagonists is already well discussed (Amadio et al., 2002; Cavaliere et al., 2003), even if down-regulation of receptors may also occur in the presence of specific agonists, as in the case of norepinephrine (Hillman et al., 2005). For any given antagonist, the rank order potency for inhibition of P2 receptor actions strictly depends on conditions such as the use of endogenous versus transfected receptors, the cell type or tissue adopted, the in vitro or in vivo experimental conditions, the presence of divalent cations, and the ionic strength of the buffer in use, to name a few. Although TNP-ATP inhibits $P2X_2$ receptor at a concentration that is 1000-fold higher than that inhibiting $P2X_1$ subtype (Khakh et al., 2001; Virginio et al., 1998), in our work we cannot exclude the participation, at least in part and at least in principle, of $P2X_2$ receptors in the actions inhibited by TNP-ATP. The involvement of $P2X_3$ or $P2X_{2/3}$ receptors (inhibited by TNP-ATP at concentrations comparable with those working on $P2X_1$) is instead probably excluded, because $P2X_3$ is apparently not up-regulated (Cavaliere et al., 2002), but eventually inhibited during an ischemic insult. Nevertheless, we performed experiments using lower doses of TNP-ATP, demonstrating that the concentration-response curves for prevention of both cell death and

modulation of $P2X_1$ receptor were very similar, thereby reinforcing the involvement of the $P2X_1$ subtype in ischemic actions. In addition, TNP-ATP only weakly prevents the upregulation of $P2X_2$ receptors by ischemia. Moreover, our results are consistent with the general pharmacological neuroprotective effect evoked by several antagonists of P2 receptors particularly against OGD-induced cell death (Cavaliere et al., 2001, 2003, 2004b; Melani et al., 2006).

Because we also proved here that maximal $P2X_1$ protein expression anticipated maximal neuronal loss, we do not exclude that expression and function of $P2X_1$ receptor might potentially trigger and/or boost cell death; consequently, the time window connecting these events might become fundamental for neuroprotective strategies. This hypothesis is in accordance with data by Rodrigues et al. (2005), establishing that activation especially of $P2X_1$ protein among P2X receptors modulates the release, for example, of glutamate, inducing further membrane damage and neuronal death. Our results suggest that induced expression of $P2X_1$ during OGD might take place as part of a wider purinergic network upregulation. First, the abundant ATP outflow occurring during an ischemic event is compatible with P2 receptor activation (Neary and Kang, 2005); second, a defined hierarchy in ligand binding affinity subsists in P2X receptor activation by extracellular ATP (the EC_{50} for ATP is 7.0 on $P2X_1$ receptor, 5.4 on $P2X_4$ receptor, 5.3 on $P2X_2$ receptor, and 3.4 on $P2X_7$ receptor); and third, $P2X_2$, $P2X_4$, and $P2X_7$ are also induced by OGD, although with diverse cellular and/or subcellular localization. Although within different binding affinities, time frames, and cellular specializations, different receptor subtypes (making up at least the $P2X_{2,4,7}$ receptors) would be simultaneously be recruited and combined on the same cell membrane in a sort of receptor web (Volonté et al., 2006) to sustain the OGD noxious events and to function as sensors/propagators of deregulated neuronal activity and ischemic relapse.

In conclusion, with this work we uncovered the neuroprotective action of the P2 receptor antagonist TNP-ATP, we extended to the $P2X_1$ subtype the repertoire of ionotropic purinergic receptors known to be involved in ischemic cells death, and we contributed to elucidating the molecular mechanisms finely involved with the OGD pathological insult. Although TNP-ATP as a polar compound would not easily pass through the blood-brain barrier, thus limiting its in vivo use as a neuroprotector, it might still inspire the synthesis of novel, similarly effective compounds.

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