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ORIGINAL ARTICLE

P2X3 receptor expression by HEK cells conditions their survival

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Abstract On nociceptive neurons, one important mechanism to generate pain signals is the activation of P2X3 receptors, which are membrane proteins gated by extracellular ATP. In this work, we have studied the recovery of recombinant P2X3 receptor expression in human embryonic kidney (HEK) cells. Our data demonstrated that HEK cells were not permissive for stable P2X3 expression, since the significant time-dependent cell loss. In vivo treatment with P2X3 receptor antagonist limited the effect. The expression of a single P2X3 point mutant Y393A, also largely accelerated cell death. We suggest the requirements of a permissive intracellular molecular machinery for appropriate receptor expression. The present report suggests that despite HEK cells are often used as recombinant expression system for the study a variety of receptors function, they represent a limiting permissive environment for P2X3 receptors.

Keywords ATP · Cytotoxicity · Apoptosis · Signaling

Abbreviations

α,β-MeATP	α,β-Methyleneadenosine 5'-triphosphate
AU	Arbitrary unit
HEK	Human embryonic kidney
WT	Wild-type

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Introduction

P2X receptors are nonselective cationic channels gated by extracellular ATP and are expressed by a variety of mammalian cell types including neurons, glia, epithelial cells, and smooth muscle cells [1]. Ample evidence indicates that specific subsets of P2X receptors are involved in processing pain signals at peripheral and spinal level.

In particular, the expression of the P2X3 subtype is very abundant in non-peptidergic small-diameter dorsal root ganglion (DRG) neurons, which are associated with transduction of pain signals via P2X3-dependent nociception [2]. In vivo studies show that the activation of P2X3 ATP receptors contributes to acute nociceptive behavior, hyperalgesia and allodynia [3, 4]. Among the seven subtypes of P2X receptor, the C-terminal domain is highly variable and shares few homology sequences. The molecular mechanisms responsible for the P2X3 receptor activity remain incompletely understood, although recent data have indicated their function to be upregulated or downregulated by their phosphorylation state [5].

To dissect out such mechanisms, it is often useful to employ recombinant receptors expressed by cell lines constitutively devoid of them like human embryonic kidney (HEK) cells [6–8], which nevertheless are highly variable in terms of receptor repertoire expression [7]. Since HEK cells may release considerable amounts of ATP [9, 10], and culture procedures and cell density may contribute substantially to it, P2X3 receptors expressed by HEK cells may be harbored in a nonpermissive environment, because these cells may not possess certain intrinsic regulatory checkpoints such as neuronal proteins or calcium buffers. In order to test this hypothesis, we compared, on HEK cells, the consequences of wild-type (WT) or Y393A-mutated P2X3 receptor expression, because the latter lack a modulatory tyrosine residue at the C-terminal

domain critical to control receptor function negatively [11]. The present report describes that HEK cells tolerated a rather limited permissive level of receptor expression as overexpression of P2X3 receptors readily produced cytotoxicity.

Methods

Cell culture and reagents

HEK-293T cells were routinely cultured in DNEM medium plus 10% FCS. Cell transfection of plasmids pCDNA3-P2X3, pCDNA3-P2X3-Y393A encoding rat WT or mutated P2X3 receptors [11], was performed with calcium phosphate method [12]. pEGFP plasmid (Clontech, Mountain View, CA, USA) was used as control. Cells were analyzed 24, 48, or 72 h after transfection. P2X3 receptors antagonist A-317491 (10 μM, Sigma, St. Louis, MO, USA [13]) was applied 24 h before experiment.

Western blot and immunofluorescence

For protein analysis, protein were extracted in a buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 2 mM EDTA plus protease inhibitors (Complete, Roche Diagnostics, Monza, Italy) and separated on 10% polyacrylamide gel. For immunoblotting anti-P2X3 (1:300; Alomone, Jerusalem, Israel) and anti-Actin antibodies (1:1000, Sigma) were used. Signals were detected with ECL (GE Healthcare, Waukesha, WI, USA) and quantified using Scion Image Software (Maryland, USA).

For immunofluorescence, cells were fixed in paraformaldehyde 4% and processed with anti-P2X3 antibody (1:300, Alomone) and anti-active caspase 3 antibody (1:100, Cell Signaling, Danvers, MA, USA). Immunofluorescence reactions were visualized using suitable AlexaFluor-conjugated secondary antibodies (1:500; Invitrogen, San Giuliano Milanese, Italy), while nuclei were counterstained with DAPI (1:1000, Sigma). Antibodies used for immunofluorescence were validated by Western blot of pCDNA3-P2X3 receptor-transfected lysates [11] as well as of rat primary DRG or mouse trigeminal neurons [14]. Cells stained with secondary antibodies only showed no immunostaining. Quantitative analysis was obtained with MetaMorph software (Molecular Devices, Downingtown, PA, USA).

Results

P2X3 receptor expression in HEK cells was activity-dependent

Recombinant transient expression of the green fluorescent protein (GFP) reporter with ATP-gated P2X3 receptors in HEK cells was used to study the effectiveness of this protocol on such cells. Heterologous gene expression is intrinsically associated to large protein expression, not regulated by naive transcription factors and gene expression control mechanisms. Nevertheless, recombinant expression of GFP has no impact on cell physiology. As shown in Fig. 1A, at different times (24, 48, and 72 h) after transfection, the yield of DAPI-labeled cells expressing P2X3 receptors was significantly lower than the one observed with the control protein GFP alone (3% vs. 17% after 24 h; 0.8% vs. 7% after 72 h, $n=3$ independent experiments, $p<0.05$). The histograms of Fig. 1A indicate that, despite the gradual disappearance of GFP-positive cells, the loss of P2X3 immunoreactivity was intense, making it almost undetectable. This effect was related to P2X3 receptors activity, since 24-h incubation with the specific P2X3 receptor antagonist A-317491 (10 μM [13]) was sufficient to significantly increase the number of P2X3-immunopositive cells (35% rise) 48 h after transfection, with respect to transfections maintained in the absence of A-317491 ($n=3$, $p<0.05$, Fig. 1B). A-317491 alone had no effect on control GFP HEK cells (not shown). This result was confirmed by higher P2X3 signal in Western blots of lysates probed with a specific anti-P2X3 receptor antibodies ($n=3$, $p<0.05$, Fig. 1C).

These data suggested that survival of HEK cells was undermined by P2X3 receptors presumably activated by extracellular ATP.

Expression of P2X3 was controlled by Y393A

To test if P2X3 receptor function was actually involved in the control of P2X3 expression, we used the P2X3 mutant Y393A that is characterized by higher receptor efficacy because of lack of a tyrosine residue necessary to produce negative control via CSK-mediated phosphorylation [11]. By comparing the number of cell expressing WT P2X3 and Y393A P2X3 receptors both in immunofluorescence and Western blot experiments (Fig. 2), we observed that the expression of Y393A was accompanied by a significant lower cell survival with respect to WT P2X3-expressing cells (Fig. 2A, B). Nonetheless, this deleterious action by the Y393A mutant was prevented (Fig. 2A, B) by the application (24 h) of the antagonist A-317491 (10 μM; $n=3$, $p<0.05$). Our data suggested that the 393 residue of the P2X3 receptor was important not only to control the expression but also to determine the viability of transfected HEK cells.

P2X3 expression in HEK cells was associated to caspase 3

To further support the role of P2X3 receptors in the cytotoxicity of HEK cells, we tested the activation of caspase 3, a known marker of apoptosis previously shown to be involved also in P2X7 receptor-mediated cell death [15]. On

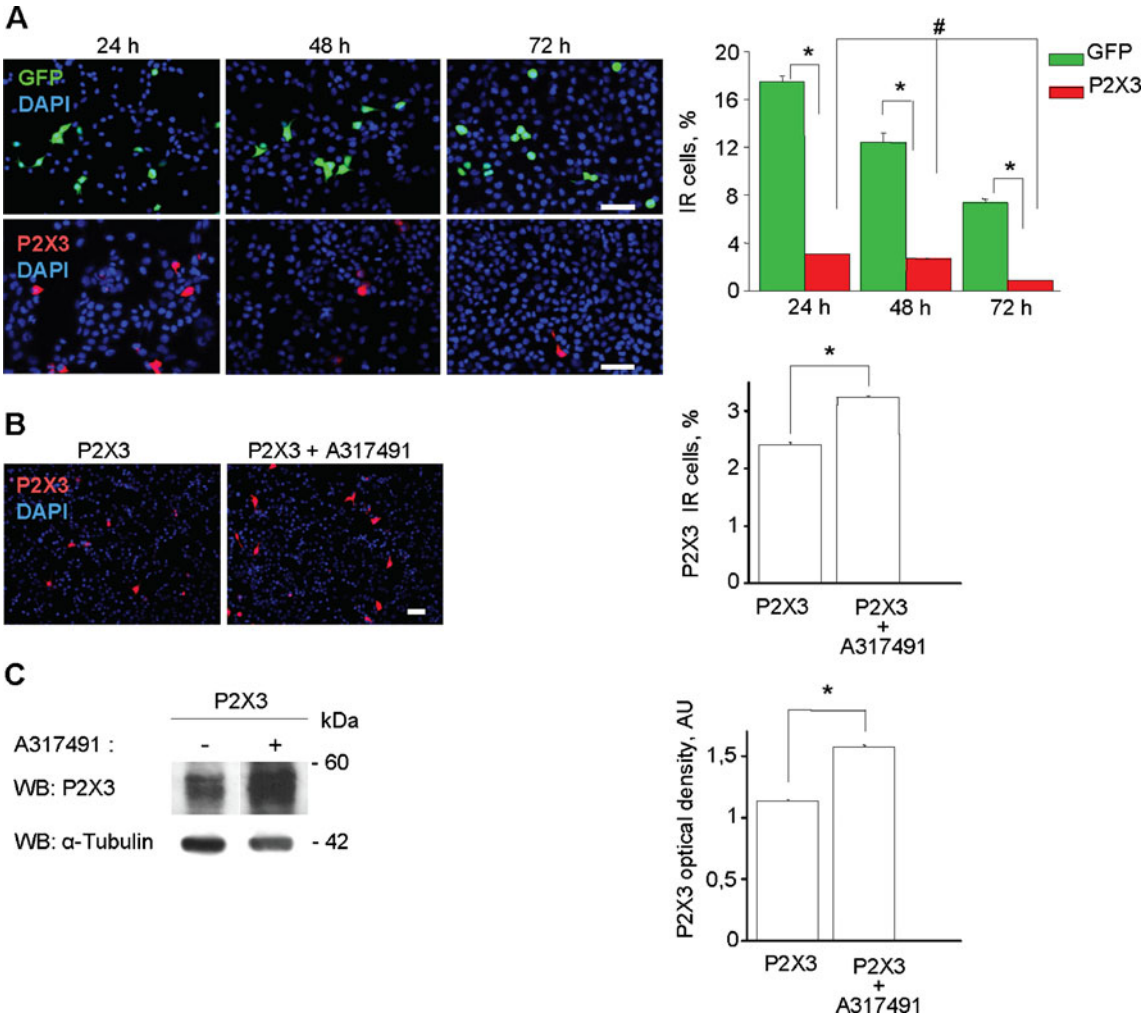

Fig. 1 P2X3 expression was time-dependent and enhanced by A-317491 antagonist. **A** Example of immunofluorescence microscopy photographs of HEK cells transfected with P2X3 (*red*) and GFP (*green*) analyzed at different times (24, 48, and 72 h) after transfection using anti-P2X3 antibodies. Nuclei are counterstained with DAPI (*blue*). Histograms (*right*) quantify the effect. Bar: 50 μm. Histograms quantify the effect, expressed as immunoreactive cells over cell nuclei. **B** Example of immunofluorescence experiments performed after 48 h from P2X3 transfection, in control and in the presence of P2X3 receptors antagonist A-317491 (10 μM, added 24 h before analysis). Histograms quantify the effect. Note how A-317491 protected the cell loss (35% increase; $n=2$; $p<0.05$). **C** Example of Western blot of P2X3-transfected cell extracts from control and from A-317491-treated cells, probed with anti-P2X3 receptors antibodies to visualize total P2X3 protein content. α-Tubulin was used as gel loading control. Histograms quantify the effect, expressed in optical density normalized over total tubulin content

HEK cells expressing P2X3 receptors, a high degree of colocalization of P2X3 receptor immunopositivity and the active form of caspase 3 (labeled by a specific antibody) was observed (Fig. 3; $n=3$, $p<0.05$). This phenomenon was never observed in cells expressing the control plasmid GFP (see Fig. 3, first raw and histograms) and it was significantly reduced when P2X3-expressing cells were incubated with A-317491 antagonist (Fig. 3, $n=3$, $p<0.05$). These data indicated that overexpression of P2X3 receptors in HEK cells induce toxic effects with consequent limited recovery of P2X3-expressing cells.

One interesting issue is the specificity of the effect we observed in P2X3-transfected cells. One possibility is that the cell context may confer specific accessory proteins that might be necessary for proper receptor function. To specifically address this point, we used rat osteosarcoma cell line ROS17/2.8, insensitive to ATP, ADP, and UTP [16]. After transfection, in comparison with HEK cells, P2X3 immunopositive ROS17/2.8 cells did not show any evident sign of morphological alteration (Fig. 4).

Discussion

To the best of our knowledge, this is the first report of P2X3 receptor-dependent cytotoxicity of HEK cells. ATP-mediated

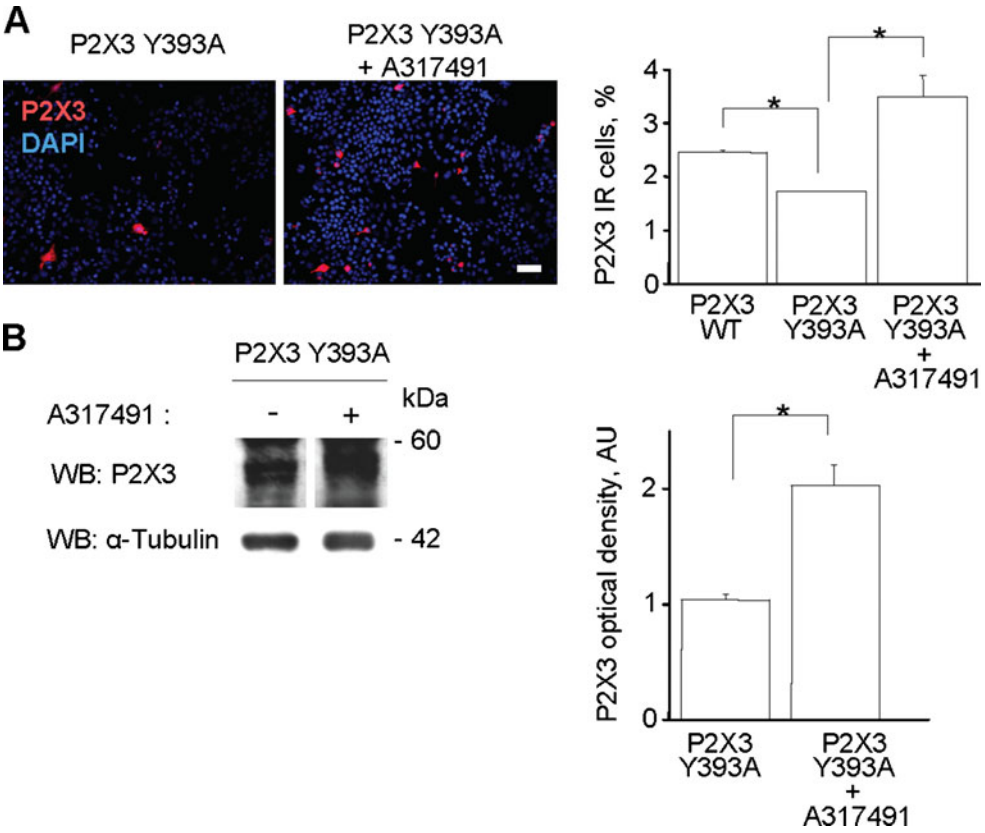

Fig. 2 Expression of P2X3 mutants was controlled by P2X3 Y393A amino acid site. **A** Example of immunofluorescence microscopy photographs of HEK cells transfected with P2X3 Y393A (*red*) in control and after A-319471 application, using anti-P2X3 antibodies. Nuclei are counterstained with DAPI (*blue*). Histograms (*right*) quantify the effect. Bar: 50 μm. Histograms quantify the effect. **B** Example of Western blot of P2X3 Y393A-transfected cell extracts from control and from A-317491-treated cells, probed with anti-P2X3 receptors antibodies to visualize total protein content. α-Tubulin was used as gel loading control. Histograms quantify the effect, expressed in optical density over total tubulin content. Note how P2X3 receptors antagonist increases P2X3-Y393A protein recovery. $n=3$; $p<0.05$

cytotoxicity has been demonstrated for P2X7 receptors of immune cells [17], since such receptor activity stimulates Ca2+ influx, membrane depolarization, and formation of large plasma membrane pores with consequent induction of apoptosis [18]. Caspase 3 activation and apoptosis morphological features are also reported following other P2X receptor stimulation activation, in particular, the P2X4 subtype [19].

Even if high ATP concentrations can induce apoptosis in several models [20], in physiological conditions, this effect is usually limited by tissue-specific molecular rescue elements. For instance, a protective anti-apoptotic mechanism, namely, nuclear factor of activated T cells complex 1, NFATc1, is found to be associated to P2X7 receptor activation in lymphoid cells [21]. Despite large amounts of ATP released during acute injury and inflammatory conditions [2], ATP-mediated caspase activation in sensory neurons expressing P2X3 receptors has not been reported. Likewise, chronic, intense pain states are not accompanied by neurotoxicity of sensory neurons. In physiological conditions ATP concentration are kept low by ecto-ATPases and therefore neurotoxity appear unlikely. In addition, adenosine has neuroprotective activity. Nevertheless, in pathological conditions (i.e., chronic pain models) abundant expression of P2X3 receptors in sensory neurons may occur, a fact that which might contribute to hyperalgesia, as well as neuropathic pain.

We posit that various cell-dependent mechanisms probably contributed to the toxicity of HEK cells. First, P2X3 receptors expressed by HEK cells show less desensitization after agonist application [22], thus possessing an impaired process to constrain the length of receptor activation. Second, it is possible that a neuronal specific regulatory mechanism limits the onset of P2X3 receptors cytotoxicity. In particular, we have recently described that, in mouse sensory neurons, the correct ratio of phosphatase and kinases (such as Csk and Cdk5) is crucial to ensure the correct function of P2X3 receptors [11, 23, 24]. We do not know the relative activity of these enzymes in HEK cells, but we have consistently observed that the membrane currents generated by P2X3 receptors on HEK cells are usually much larger than those recorded with native P2X3 receptors of sensory neurons [14–22]. Mutating the tyrosine 393 (essential for the Csk function on P2X3 receptors receptors) enhanced the cytototoxicity of P2X3 receptors in accordance with this notion.

Extracellular Ca^{2+} influx via transfected P2X3 receptor channels contribute substantially to membrane currents changes and to vary intracellular Ca^{2+} concentration in HEK cells [25]. Furthermore, cell-specific proteins like Ca^{2+} binding buffers may be associated to proper expression and function of P2X3 receptors and control their responses. For instance, the calcium buffer protein VILIP has already

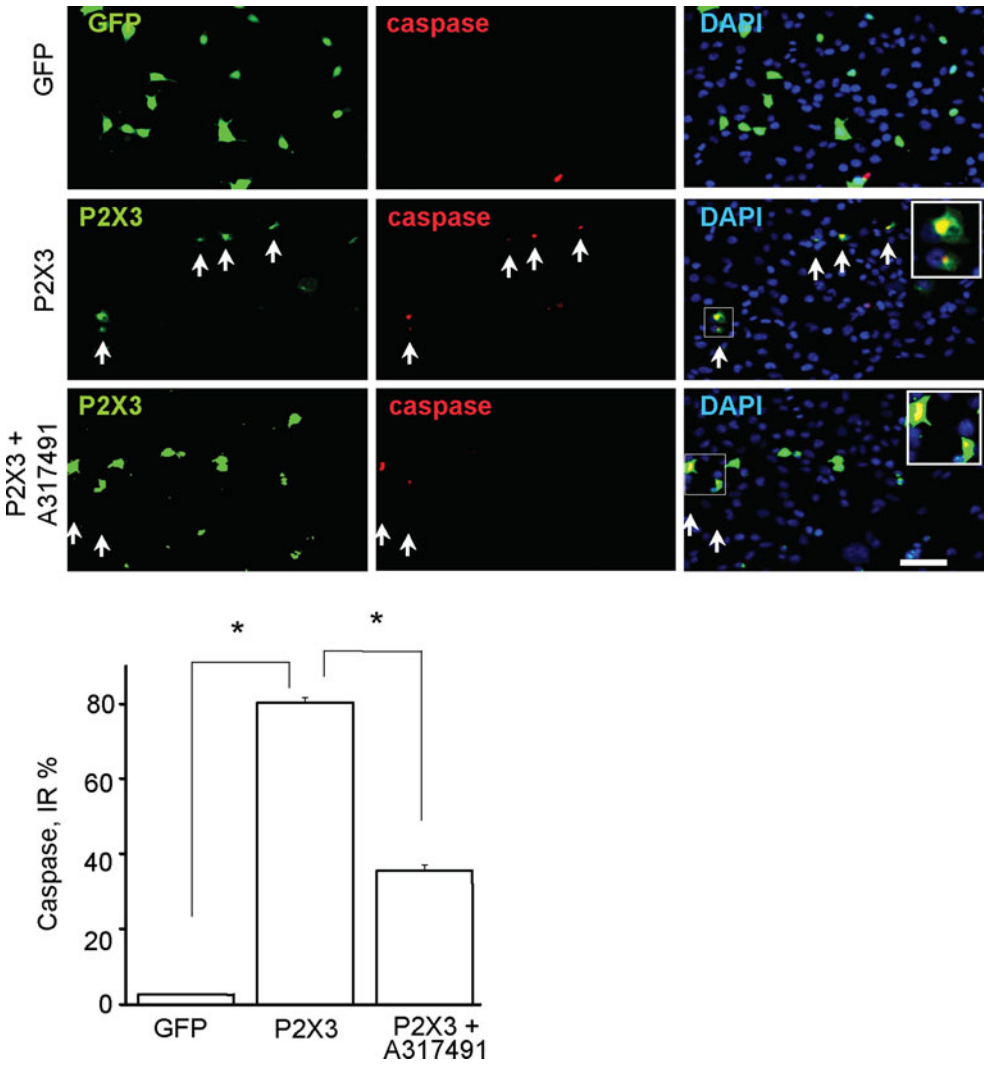

Fig. 3 P2X3 expression in HEK cells was associated to caspase 3 activation. Example of immunofluorescence microscopy photographs performed with antibodies against P2X3 (*green*) and anti-active caspase 3 (*red*), as one of the hallmark of ongoing apoptosis. Control GFP-expressing cell are shown in *green* and were not associated to caspase activation (*first upper row*). Bar: 50 μm. Histograms quantify the effect, expressed as caspase-positive cells over a total of P2X3 immunopositive cells. Note how antagonist A-317491 protects from caspase activation. $n=3$; $p<0.05$

been proposed to control calcium influx through P2X2 receptors [26]. Future studies are necessary to find out the detailed molecular mechanisms responsible for enhanced death of HEK cells expressing P2X3 receptors. In particular, single amino acids of P2X3 receptors can be crucial to control cell-specific protein–protein interaction important for expression and function of P2X3 receptors at membrane level. Absence of citotoxicity in ROS17/2.8 cells, suggest that the cellular context is highly important for proper expression of P2X3 function. One possibility is also that the proper P2Y-P2X repertoire expressed by single cell types is important in further consequences to ATP exposure, an issue that nevertheless requires further investigations.

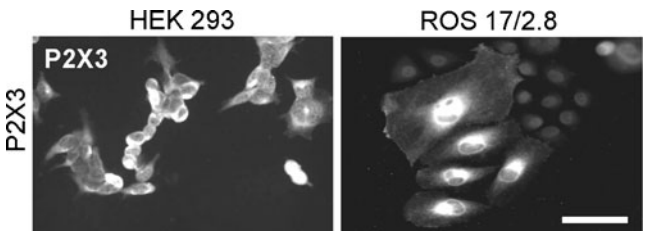

Fig. 4 Expression of P2X3 receptors in ROS 17/2.8 cells. Example of microscopy photographs of transient P2X3 receptors expression in HEK-293 cells (*left*) and in rat osteosarcoma cell line ROS17/2.8 (*right*) after immunofluorescence with anti-P2X3 antibodies. Note that, in contrast to HEK cells, P2X3-immunopositive ROS17/2.8 cells did not show relevant morphological alterations. Scale bar: 50 μm

The present data suggest that, in addition to ionotropic glutamate receptors that are canonically associated with excitotoxicity of neurons [27], it is possible to confer cytotoxic action to P2X3 receptors when they are expressed by a host system like HEK cells.

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