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Up-regulation of $P2X_7$ receptor-immunoreactivity by *in vitro* ischemia on the plasma membrane of cultured rat cortical neurons

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ABSTRACT

Mixed neuronal/astrocytic cortical cell cultures of the rat were incubated for 2 or 12 h under normoxic or ischemic conditions. Subsequent flow cytometric analysis with an anti-$P2X_7$ receptor antibody directed against an extracellular epitope indicated the up-regulation of these receptors at the plasma membrane by 12 h of ischemia. Labelling of MAP-2 immunopositive neurons by an anti-$P2X_7$ antibody directed against a C-terminal epitope, documented the selectivity of the ischemia-induced increase in receptor-density for the neuronal population. By contrast, staining of GFAP immunopositive astrocytes by the same anti-$P2X_7$ antibody excluded any effect of ischemia on the astrocytic density of $P2X_7$ receptors. The ischemic up-regulation of neuronal $P2X_7$ receptors is in perfect agreement with the previously reported facilitation of transmitter release from the GABAergic non-pyramidal cell type in such cultures [K. Wirkner, A. Köfalvi, W. Fischer, A. Günther, H. Franke, H. Gröger-Arndt, W. Nörenberg, E. Madarasz, E.S. Vizi, D. Schneider, B. Sperlagh, P. Illes, Supersensitivity of $P2X_7$ receptors in cerebrocortical cell cultures after *in vitro* ischemia, J. Neurochem. 95 (2005) 1421–1437].

$P2X_7$ receptors are ATP-gated cationic channels, which on prolonged or repetitive agonist activation induce rapid membrane and mitochondrial morphological changes, cytoskeletal rearrangement and ultimately cell death [25,17]. They are thought to be primarily expressed on cells of hemopoietic origin, where $P2X_7$ receptors participate in immune responses, cell proliferation, cell death, and bone formation/reabsorption. These receptors are also present at immunocompetent cells of the central nervous system such as microglia and astrocytes [21]. In astrocytes, they appear to mediate both communication within the glial network by Ca^{2+} waves and communication between glial cells and neurons *via* gliotransmitters [23]. In addition, their activation leads to apoptotic and necrotic as well as proliferative changes in astrocytes [8]. Interestingly, similar changes were observed also on microglia [2].

Recently, there is a major dispute about the existence of neuronal $P2X_7$ receptors. The major counterargument is that two groups of authors reported pseudoimmunoreactivity for this receptor-type in hippocampal neurons of $P2X_7^{-/-}$ mice in spite of confirming their absence in peripheral immune cells and the submandibular gland ([13,20]; but see Ref. [10]). However, functional studies by means of selective agonists and antagonists strongly support a $P2X_7$ receptor-mediated transmitter release from the terminals of central glutamatergic, GABAergic and noradrenergic neurons activated both by exogenous agonists and endogenously accumulating ATP [23,1]. This ATP may be released by cellular damage caused by traumatic injury or metabolic limitation during ischemic insults. $P2X_7$-immunoreactivity in the knockout mice was suggested to be due to the presence of a $P2X_7$-like and still functional protein in the brain, which is identical with the genuine $P2X_7$ receptor regarding the epitopes recognized by the antibodies, but differs in the sequence disrupted by the genetic manipulation [18,14]. In the present study, we have investigated the up-regulation of $P2X_7$ receptor-immunoreactivity by *in vitro* ischemia in cortical mixed neuronal/astrocytic cultures and identified the cell type involved.

Cell cultures were prepared from rat fetuses at gestational day 16 and grown as described earlier for 14 days *in vitro* (14 DIV; [26]). *In vitro* ischemic conditions were created by treating these cultures with glucose-free medium containing DMEM, supplemented with F12, 5% fetal calf serum (FCS), 15 mM HEPES, 50 μg/ml gentamicin (Invitrogen, Karlsruhe) in a N_2 incubator with 6.8% CO_2 and 3% O_2 at a temperature of 37 °C in a humidified atmosphere. Normoxic conditions were generated by keeping the cells in a cell culture incubator at 37 °C and 6.8% CO_2 in humidified atmosphere and 20 mM glucose-containing cell culture medium. The effects of an ischemic incubation (oxgen/glucose-deprivation; OGD) for 2 or 12 h of duration were compared with the respective normoxic counterparts.

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Cells were detached with accutase (0.5 ml per culture dish; PAA Labs, Pasching, Austria) and 5×10^5 to 1×10^6 cells were used in one sample. For membrane staining, pellets obtained by centrifugation were resuspended in 50 μl phosphate buffered saline (PBS) containing 2% FCS. Cell suspensions were incubated with 1 μl of a polyclonal rabbit-anti-mouse $P2X_7$ receptor antibody (FITC-labelled antibody directed against the extracellular residues 136–152 of the mouse $P2X_7$ receptor; Alomone Labs, Jerusalem, Israel) for 1 h on ice and in the dark (green fluorescence). The cells were then washed twice with PBS containing 2% FCS.

For intracellular staining cells were fixed and permeabilized using the Cytofix/Cytosperm solution from Becton Dickenson (BD) Biosciences (Heidelberg, Germany) following the manufacturer's instructions. Cells were stained in 50 μl of PERM/Wash buffer from BD Biosciences using 1 μl of a polyclonal rabbit-anti-$P2X_7$ receptor antibody (directed against the intracellular C-terminal residues 576–595 of the rat $P2X_7$ receptor; Alomone Labs) combined with 0.5 μl of a monoclonal mouse-anti-GFAP (glial fibrillary acidic protein) antibody (Sigma, Saint Louis, USA) or 1 μl of a monoclonal mouse-anti-MAP-2 antibody (Chemicon, Millipore, Schwalbach, Germany) for 1 h on ice. Then, cells were washed twice and incubated in 50 μl PERM/Wash buffer containing a goat-anti-rabbit-IgG, Cy-3 labelled (red fluorescence; intra-$P2X_7$) and a donkey-anti-mouse-IgG, Cy-2 labelled (green fluorescence; MAP-2 and GFAP) secondary antibody, both from Jackson Labs (Bar Harbor, Maine, USA) for 1 h on ice

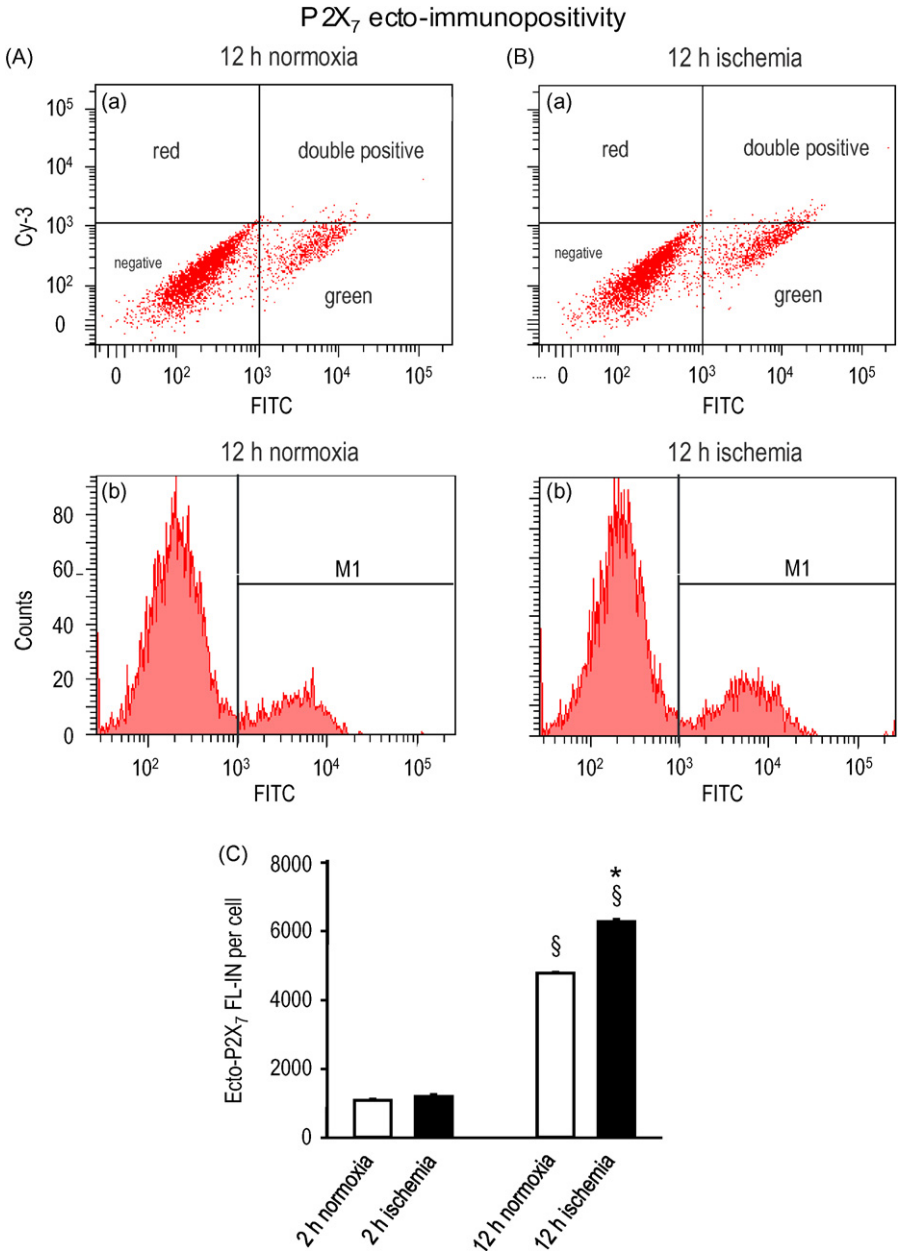

Fig. 1. FACS analysis of $P2X_7$ receptor membrane expression at cultured neuronal/astrocytic cells of the rat cerebral cortex. These cultures were subjected to normoxic or ischemic incubation for 2 or 12 h. Cells were detached using accutase. To detect $P2X_7$ receptors, cells were stained with a FITC labelled antibody (green fluorescence) directed against an extracellular epitope of the $P2X_7$ receptor (ecto-$P2X_7$). Dot blots (a) and FITC histograms (b) are shown both for the normoxic (A) and ischemic (B) incubations for 12 h. Green fluorescent cells specifically bind the ecto-$P2X_7$ antibody at their plasma membrane. The horizontal line above the histograms (M1) indicates the cell population included into further evaluations out of the total number of cells. The FITC fluorescent intensity (FL-IN) of these cells is shown after the different treatment protocols (C). $^*P < 0.05$, statistically significant difference between empty and filled columns. $^§P < 0.05$, statistically significant difference from the respective treatment for 2 h.