ATP INDUCES SYNAPTIC GENE EXPRESSIONS IN CORTICAL NEURONS: TRANSDUCTION AND TRANSCRIPTION CONTROL VIA $P2Y_1$ RECEPTORS

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Molecular Pharmacology

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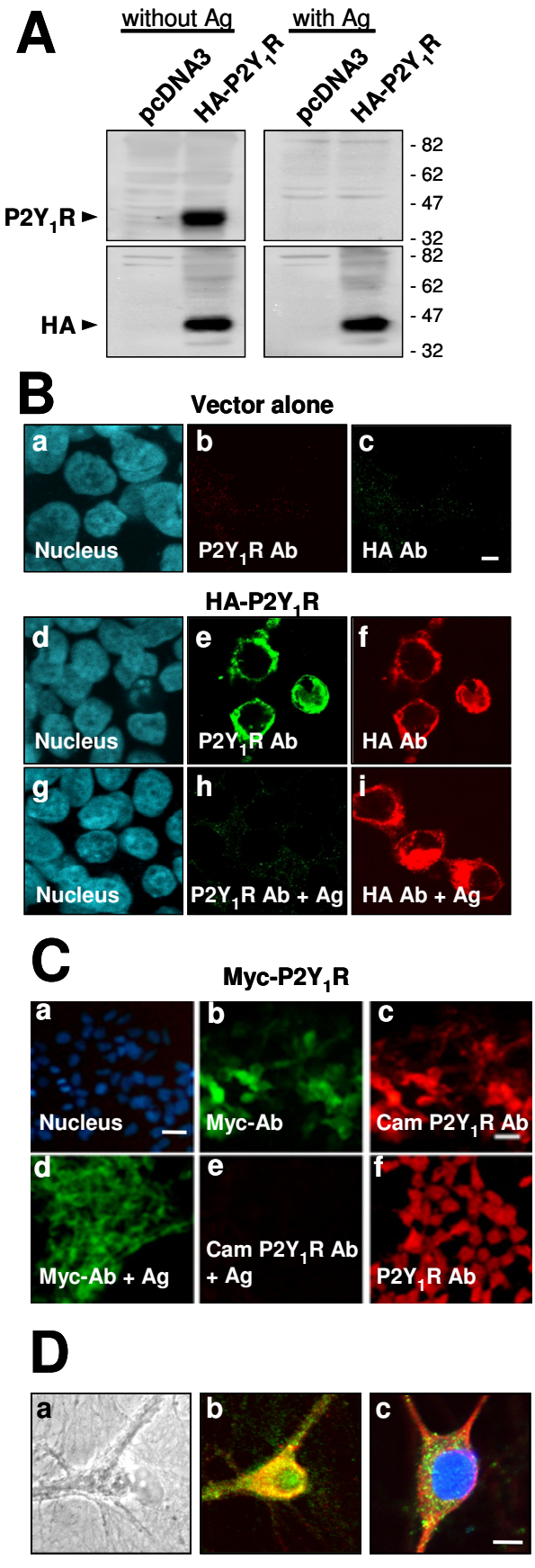
SUPPLEMENTAL DATA S1.



S1. Testing the specificity of the anti-P2Y$_1$R antibody used.

All methods mentioned here were as specified in Materials and Methods of the main text. All the Figure panels cited below are in Fig. S1. The "anti-P2Y$_1$R antibody" here is the Alomone antibody used for identifying P2Y$_1$R in the main text, i.e. in Figs.1C, D, E and 2A there. (In Fig.3 there, transfected P2Y$_1$R was recognised, instead, by a labeled antibody to an N-terminal Myc peptide tag attached to it).

S1 Supplemental Results.

Firstly, the P2Y$_1$R receptor in heterologous expression in HEK 293T cells was used. The P2Y$_1$R protein was transiently expressed there equipped with an N-terminal tag of a hemagglutinin peptide (HA-P2Y$_1$R) and that was detected in parallel using an antibody to the HA epitope. The anti-P2Y$_1$R antibody reaction was shown (Fig. S1): (i) after extraction of those cells and immuno-blotting (panel A), to stain a single band of the predicted protein size and corresponding exactly to the HA-tagged protein band in the same lane; (ii) in immuno-cytofluorescence in confocal microscopy, to stain the cells strongly (green), and identically to the anti-HA antibody (red) staining (panel B, a-f); (iii) to undergo full block by the P2Y$_1$R antigenic peptide of the staining in the immuno-blots (panel A) and on the cells (panel B, g-i), but showing no block by that peptide of the corresponding cell staining by the anti-HA antibody (panel B, i). The confocal plane passes centrally through the 3 cells in panels Be and Bf, showing membrane P2Y$_1$R, with one of these cells (smaller) showing also some intracellular receptor (presumed in transit). In another sample (Bi), in 2 of 3 cells shown a higher plane captures the receptor-rich upper cell surface staining (using anti-HA). Hence in immuno-blotting and in immuno-cytofluorescence the anti-P2Y$_1$R antibody which we used was specific in transfected HEK cells. However, that does not establish specificity in brain neurons, where additional constituents might in theory cross-react. A further test of the specificity of this antibody became feasible, however, because late in the present work a new anti- P2Y$_1$R antibody became available for comparison. From an antibody set recently prepared by our laboratory in Cambridge, an anti-P2Y$_1$R antibody (here termed Cam- P2Y$_1$R antibody) directly-labeled by conjugation to the cyanine dye Cy3 *or* to fluorescein (via its iso-thiocyanate, FITC), was validated by showing that its robust staining on endogenous P2Y$_1$R in brain neurons is totally eliminated in P2Y$_1$ gene knock-out mice (details to be published elsewhere). We then compared the Alomone and Cam- P2Y$_1$R antibodies, firstly

using a HEK 293 cell line stably expressing at moderate levels $P2Y_1R$ N-terminally tagged with the Myc peptide (Myc-$P2Y_1R$ R cells: Choi et al., 2008). In the serum/BSA medium these cells (permeabilized) were stained identically by the Alomone antibody, or the Cam-$P2Y_1R$ antibody, or an anti-Myc antibody. These cells were viewed in epi-fluorescence microscopy: this was done to view the cells from above, showing again the considerable receptor presence on the upper surface. Representative samples are shown in Fig. S1, panel C: **a-c,** a given field of clumped cells (in epi-fluorescence microscopy), showing the nuclei (DAPI stain, **a**), or stained (**b**) for Myc with FITC-conjugated anti-Myc antibody, green (1:100), or stained (**c**) with the Cy3-conjugated Cam-$P2Y_1R$ antibody, red (1:100); **d, e,** the peptide antigen of the Cam-$P2Y_1R$ antibody was present, with **d** stained for Myc as in **b,** and **e** stained with the Cam-$P2Y_1R$ antibody; **f,** another sample of the same cells is stained (without the antigen) with the Alomone anti-$P2Y_1R$ antibody (1:200); Bar: 25 μm . The correspondence of the staining by the directly-labeled antibody (of either origin) with the anti-tag stain in both the B and the C panels shows that the antibody we use in the main text is specific for the $P2Y_1R$ in these cells.

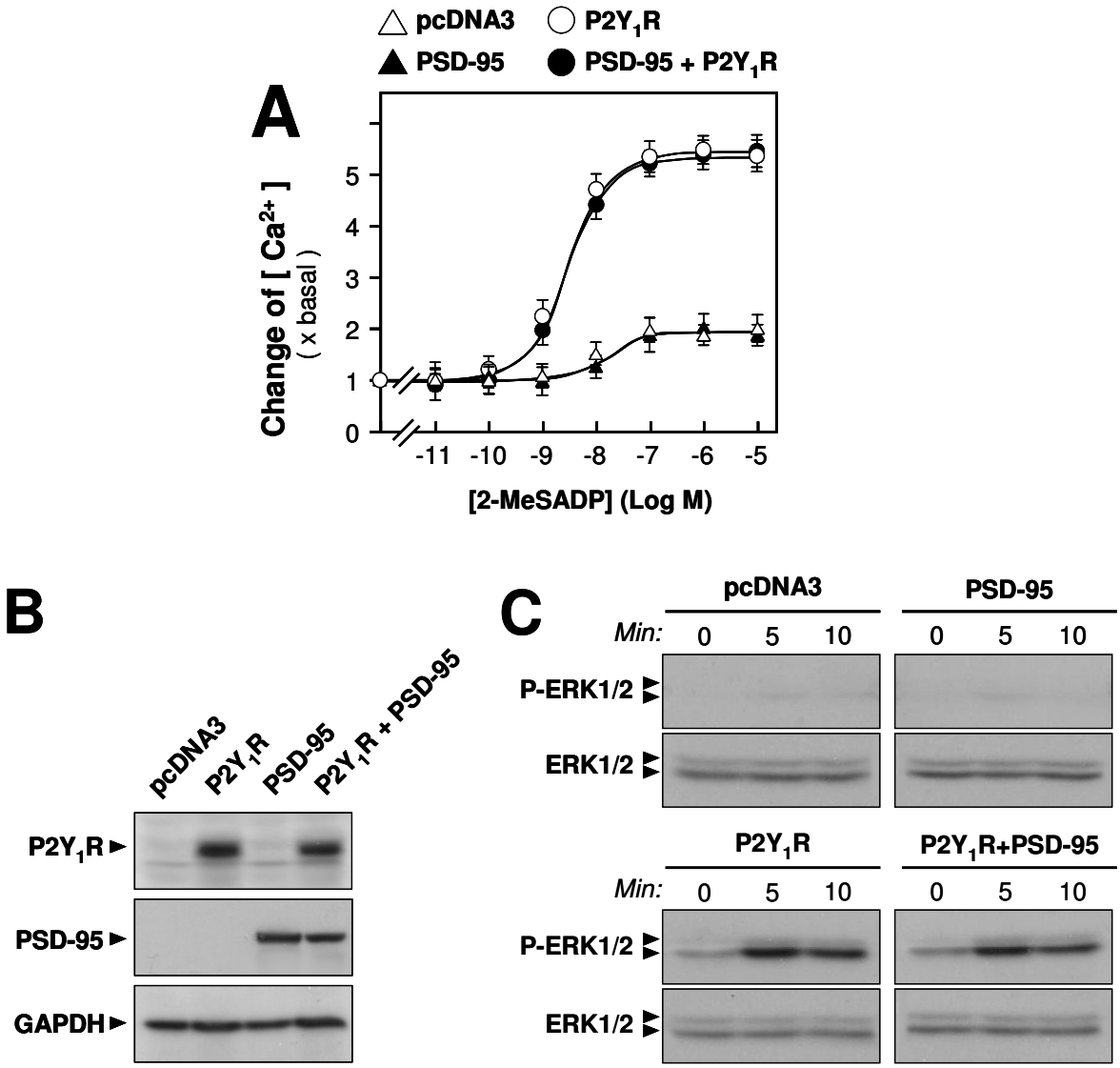
This test was then extended to the reaction of the Alomone antibody with native $P2Y_1Rs$ in brain neurons, to go beyond the heterologous expression used in the tests above and to use specimens equivalent to the cortex cultures which are used to study native functional $P2Y_1Rs$ in the studies to be reported below. To test the antibodies on neurons where there is independent evidence for the presence of endogenous, functional $P2Y_1Rs$, we could use rat hippocampal primary cultures. On these it has been shown in a collaborative study (Filippov et al., 2006) that functional $P2Y_1Rs$ are present on most of the pyramidal neurons, using an independent criterion, i.e. $P2Y_1R$-stimulated inhibition of a direct K^+-channel native response (the M-current) on those antibody-identified cells, by M-current recordings using $P2Y_1R$ agonists and antagonists. Comparisons of the antibodies were made using such hippocampal cultures (15 DIV, prepared as in Filippov et al., 2006). Shown in Fig. S1, panel D: **a,** In brightfield; **b,** the same cell, stained with the Alomone antibody (1:100, green) and anti-neurogranin antibody (red, pyramidal cell marker); **c,** another such cell, stained with the Cam-$P2Y_1R$ antibody (1:100, green) and anti-MAP-2 (red, neuronal microtubule marker). In **b** the top surface of the cell is viewed, with the underlying nucleus (DAPI stain, blue) well below it. In **c** the confocal plane is through the nucleus. Overall, 88% of the neurogranin-positive pyramidal neurons in these cultures stain for $P2Y_1R$. That stain is always on the cell body and on dendrites, but its extent on the dendrites varies, appearing to

increase with the differentiation of these neurons. Bar: 10 μm. (Permeabilization with Triton was applied, after fixation and prior to staining, in panels B, C, D **,** to allow any intracellular epitopes to be reacted).

Conclusion.

Staining of the $P2Y_1R$ s on the pyramidal neurons was equally and reproducibly obtained in parallel with the Cam-$P2Y_1R$ specific antibody and with the Alomone antibody used in the present studies. While this does not prove that the Alomone antibody will be specific also on intact brain sections, where additional components cross-reactive with it may be retained *in situ,* we conclude that it is, at the least, specific on the primary cultures which we use.

SUPPLEMENTAL DATA S2.



S2. Testing the influence of PSD-95 in affecting the receptor signalling of $P2Y_1R$ in transfected HEK293T cells.

We tested whether the signaling responses of $P2Y_1R$ were affected by the presence of PSD-95. HEK293T cells were transfected with pcDNA3 (mock control), PSD-95, $P2Y_1R$ or $P2Y_1R$+PSD-95, the Ca^{2+} mobilization and ERK phosphorylation were measured, using with the procedures described in the Methods section.

S2 Supplemental Results.

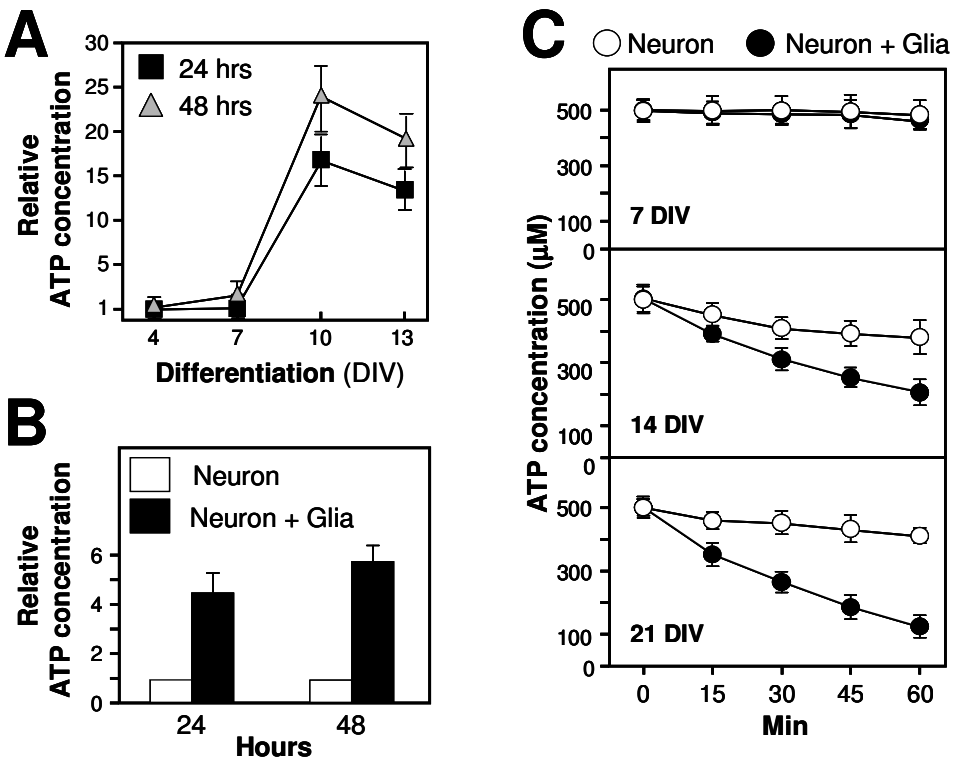
In the pcDNA3-transfected culture (opened triangles), the addition of 2-MeSADP at high concentration triggered only a low response of Ca^{2+} influx (Fig. S2A). The same results were observed in PSD-95-transfected cultures (filled triangles). When $P2Y_1R$ was expressed (opened circles), Ca^{2+} influx was dramatically induced by 2-MeSADP in a dose-dependent manner. The same high response was observed when PSD-95 was co-expressed with $P2Y_1R$ (filled circle).

For ERK phosphorylation, the transfected cultures were firstly collected to examine the expression of $P2Y_1R$ and PSD-95, each alone there (Fig. S2B). Next, the transfected cultures were serum-starved and then challenged by 2-MeSADP (1 μM) for 10 min. The results showed that a very weak response of ERK phosphorylation was observed in both control pcDNA3- and PSD-95-transfected cells (Fig. S2C). In the presence of the expressed $P2Y_1R$, the ERK phosphorylation induced by 2-MeSADP was significantly enhanced. However, there was no obvious difference of phosphorylation intensity between $P2Y_1R$ and $P2Y_1R$+PSD-95 cells.

Conclusion.

These results suggested that PSD-95 serves as a scaffold protein, in this case to concentrate $P2Y_1R$ at the plasma membrane, rather than regulating its receptor signaling.

SUPPLEMENTARY DATA S3.



S3. Net release of ATP/ADP onto cortical neurons.

S3 Supplementary Methods: Determination of ATP concentration and hydrolysis on the cultures used

ATP in the media conditioned by our cultures in various specified conditions was assayed by the luciferase method described (Ling et al., 2005). Each ATP concentration was calculated from calibration plots of ATP standards (0-100 nM or 0-500 μM). To allow for the additional release by mechanical disturbance by the (gentle) change of medium made in the first day of sampling, the additional increase over a further (undisturbed) 24 h period was taken (Fig S3, A). Determination of ecto-nucleotidase activity was by a method modified from Vollmayer *et al.* (2001). Cultures (on 35-mm plates) were washed twice and incubated in culture conditions at 37°C in medium (2 ml) containing 500 μM ATP; at intervals the hydrolysis of ATP in this culture-conditioned medium was monitored likewise.

S3 Supplementary Results.

The concentration of ambient ATP in contact with the cultured cortical neurons was measured here by the luciferase assay using medium conditioned by either neurons cultured alone or by neurons plus astrocytes. The release of ATP from the cultured neurons when alone was negligible after 7 DIV but thereafter increased and reached a near-equilibrium concentration at 12-15 DIV (when accumulated over the last 48 h; Fig S3A).

The activity of ecto-nucleotidase in the cultures was also investigated. Exogenous ATP was applied to the cultures and the level of residual ATP therein monitored with time. With neurons in the early stage of differentiation (7 DIV) the level of ambient ATP did not significantly change for at least 60 min of measurement: this situation was the same in cultures having neurons and glia (Fig. S3C). That situation was much changed with further differentiation: in neuronal cultures at 14 and 21 DIV the loss of ATP in the medium was relatively rapid when the cultures contained glial cells, but 4- to 5-fold slower in the neuronal cultures which we used in the receptor studies (Fig. S3C). Thus, in the resting state (i.e. without added ATP), the low ambient ATP level is the result of equilibrium reached between the spontaneous, constant release of ATP from the glia and neurons and its removal by the high level of surface ecto-nucleotidase activity seen at the surface of the glial cells.

We had confirmed (Figs. 1, 2 of the main text) the presence of abundant $P2Y_1R$ expression in neuronal cultures with minimal glia content. When the glia are allowed to develop normally with these neurons (Fig. S3B), the equilibrium concentration of ATP in the overlying bulk solution is much increased, to 20-25 nM after 48 h. This is in agreement with the now well-established constant release of ATP from astrocytes *in situ* in the brain (see Discussion, first section, in main text). The same glial contribution has been found in the hippocampal case, where Zhang et al. (2003) have shown that cultures of astrocytes produce, when stimulated by glutamate, a very much higher ambient ATP concentration than that from similarly-treated hippocampal neurons alone, even when the latter neurons were maintained in glia-preconditioned medium. The relationship of these findings to the effects of our treatments with ATP and derivatives is considered in the main text Discussion (first section).

The ambient ATP concentration measured in the bulk solution here (Fig. S3) would give, however, a gross under-estimation of the spontaneous $P2Y_1R$ activation at the cell membrane. Thus, using a membrane-tethered luciferase construct as a local ATP-biosensor on astrocytes, Joseph et al. (2003) deduced that there is a constant release of ATP from

specific plasma membrane subdomains on them into an unstirred surface layer there, such that ATP would be transiently available to local P2Y receptors before its destruction by surface ecto-nucleotidase or its loss by wider diffusion. In the brain regions involved the astrocytic processes are tightly wrapped onto the neurons (see Discussion, first section, in main text), which can position the neuronal P2Y receptors against those micro-domains. It was shown (Joseph et al., 2003) that the true local concentration of ATP or ADP there is at the least 20-fold that measured simultaneously in the bulk medium. In our untreated neuronal-glial cultures, since the bulk phase steady-state level noted above is 20-25 nM ATP, this would give a value of 400-500 nM in the localised surface layer. This level should be yet higher when the brain structure is intact *in vivo*, where diffusion in the extracellular space around neurons in regions such as cortex and hippocampus is much more restricted than in their primary cultures (Zhang et al., 2003). Further, the estimation ignores the rapid initial conversion of most of the ATP to the $P2Y_1R$ agonist ADP by NTPDase2, the predominant nucleotidase present (see the Discussion in the main text, para.2). That factor is important because ADP has a higher affinity than ATP, e.g. by 20-fold at human $P2Y_1R$ (Waldo & Harden, 2004). The native sensitivity in this activity of $P2Y_1R$ on rat brain neurons has been illustrated in our collaborative electrophysiological studies (Filippov et al., 2006), where applied ADPβS (which has the same potency as ADP at $P2Y_1R$: Simon et al., 2001) reacted (under adenosine receptor blockade) with EC_{50} 84 nM in a $P2Y_1R$ physiological response, namely closure of the M-type K^+ channel and the associated major increase in the firing of pyramidal neurons Further, on the rat cortical neuronal cultures we show here that 2-MeSADP in a functional assay has EC_{50} 71.3 nM at the native $P2Y_1R$. Hence, in summary, existing evidence indicates that the localized concentration of $P2Y_1R$ agonists produced at the surface of developing neurons *in situ* would be ample to activate the $P2Y_1Rs$ there.

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