

MOL #66506

**ATP INDUCES SYNAPTIC GENE EXPRESSIONS IN CORTICAL NEURONS:
TRANSDUCTION AND TRANSCRIPTION CONTROL VIA P2Y₁ RECEPTORS**

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MOL #66506

Running title: P2Y₁ receptors in central synapses

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Abbreviations: A3P5P, adenosine-3',5'-bisphosphate; AChE, acetylcholinesterase; AChR, nicotinic acetylcholine receptor; Ara-C, cytosine arabinoside; BSA, bovine serum albumin; ChAT, choline acetyltransferase; CNS, central nervous system; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle medium; ERK, extracellular signal-regulated kinase; GAPDH,

MOL #66506

glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GFP, Green Fluorescent Protein; GPCR, G-protein-coupled-receptor; HA, hemagglutinin; HBSS, Hanks' balanced salt solution; HEK 293T, human embryonic kidney 293T; IB, immuno-blot; IP, immuno-precipitation; Luc, luciferase; M1-AChR, muscarinic acetylcholine receptor M1; MAP kinase, mitogen activated protein kinase; MAP-2, microtubule- associated protein 2; 2-MeSADP, 2- (methylthio)adenosine 5'-diphosphate; NF-200, neurofilament 200; NHERF-2, Na⁺/H⁺ exchanger regulatory factor type 2; NR2, N-methyl-D-aspartate receptor 2; NTPDase-2, nucleoside-triphosphate-diphosphohydrolase-2; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PKC, protein kinase C; PRiMA, Proline-Rich Membrane Ancor; PSD, postsynaptic densities; PSD-95, postsynaptic density protein 95; P2Y₁R, P2Y₁ receptor; SDS-PAGE, sodium dodecylsulfate - polyacrylamide gel electrophoresis; TBST, Tris buffered saline/Tween-20; TPA, 12-O-tetradecanoylphorbol-13-acetate; TTX, tetrodotoxin.

Abstract

Studies in vertebrate neuromuscular synapses have previously revealed that ATP, via P2Y receptors, plays a critical role in regulating postsynaptic gene expressions. An equivalent regulatory role of ATP and its P2Y receptors would not necessarily be expected for the very different situation of the brain synapses, but we provide evidence here for a brain version of that role. In cultured cortical neurons, the expression of P2Y₁ receptors increased sharply during neuronal differentiation. Those receptors were found mainly co-localized with the post-synaptic scaffold PSD-95. This arises through a direct interaction of a PDZ domain of PSD-95 with the C-terminal PDZ-binding motif, D-T-S-L of the P2Y₁ receptor, confirmed by the full suppression of the co-localization upon mutation of two amino-acids therein. This interaction is effective in recruiting PSD-95 to the membrane. Specific activation of P2Y₁ (G-protein-coupled) receptors induced elevation of intracellular Ca²⁺ and activation of a mitogen-activated protein kinase/Raf-1 signaling cascade. This led to distinct up-regulation of the genes encoding acetylcholinesterase (AChE_T variant), choline acetyltransferase and the NMDA receptor subunit NR2A. This was confirmed, in the example of AChE, to arise from P2Y₁-dependent stimulation of a human *ACHE* gene promoter. That involved activation of the transcription factor Elk-1; mutagenesis of the *ACHE* promoter revealed that Elk-1 binding at its specific responsive elements in that promoter was induced by P2Y₁ receptor activation.

MOL #66506

The combined findings reveal that ATP, via its P2Y₁ receptor, can act trophically in brain neurons to regulate the gene expression of direct effectors of synaptic transmission.

MOL #66506

Introduction

Adenosine 5'-triphosphate (ATP) is both a transmitter and a modulator of signaling in the nervous system (Abbrachio et al., 2006; Filippov et al., 2006; Hussl and Boehm, 2006; Neary and Zimmermann, 2009). In the brain, ATP is present not only at millimolar levels in the cytosol of all cells, but is also at significant concentrations in the extracellular space around the neurons. Thus, glial cells have been shown to constantly release ATP which can modulate local neuronal activity (as discussed below), while from neurons ATP is well-known to be frequently co-released with other neurotransmitters at central and peripheral synapses. Neuronal actions of ATP in the central nervous system (CNS), both pre-synaptically and post-synaptically, have often been studied, but as short-term effects in exciting P2X ion channels or in modulating neurotransmission via the P2Y family of G-protein-coupled-receptors (GPCRs) for nucleotides (Bowser and Khakh, 2004; Hussl and Boehm, 2006; Lee et al., 2007; plus references therein). A few long-term effects of extracellular nucleotides in the CNS have also been observed (Liu et al., 2008), but analysis of the molecular mechanisms of those P2Y receptor actions has begun so far only on the astrocyte contribution (reviewed by Neary and Zimmermann, 2009), not on the neurons and synapses. However, in the peripheral nervous system, as in vertebrate skeletal neuromuscular junctions, we have previously found such a long-term action of ATP or UTP via their P2Y receptors there, namely in regulating and maintaining synaptic maturation. Thus, ATP acts as

a trophic factor to induce and maintain the expression of genes for the postsynaptic nicotinic acetylcholine receptors (AChRs) and acetylcholinesterase (AChE): P2Y₁ and P2Y₂ nucleotide receptors are localized in the synaptic region and both subtypes act to trigger that action (Choi et al., 2001a, 2003; Tung et al., 2004). The pathway to activation of the *ACHE* gene, mediated by the P2Y₁ receptor (P2Y₁R), was shown to involve protein kinase C, intracellular Ca²⁺ release and phosphorylation of extracellular signal-regulated kinases (ERKs) (Choi et al., 2003). In developing muscle, this culminates in activation of the transcription factor Elk-1 which acts on the promoter of the *ACHE* gene. For CNS neuronal synapses, the P2Y₁R has been shown to be, exceptionally in the P2Y family, widely expressed on brain neurons, as seen by strong P2Y₁R mRNA expression there (Webb et al., 1998; Moore et al., 2001; Rodriguez et al., 2005) and authenticated P2Y₁R protein and functional responses there as cited in the later section of the Results here. However, a role of any of this receptor family in the regulation of the gene expression of proteins involved directly in CNS synaptic transmission (effectors) has not previously been investigated.

Proceeding from those results on the neuromuscular synapse, here we study the rodent cerebral cortex for potential long-term actions of P2Y₁R. We find that in neurons from the developing cortex the P2Y₁R subtype is, indeed, significantly expressed and functional. Hence we show that it is in an association with postsynaptic density protein 95 (PSD-95), a marker postsynaptic protein known (Kim and Sheng, 2004) to form the major scaffold for a

set of brain receptors in excitatory synapses. Finally, we demonstrate the stimulation via P2Y₁Rs of gene expression for several transmission-effect or proteins in the cortex neurons, and map some steps in the pathway which leads to that control of their transcription.

There are some reports in the literature now on a range of molecular pathways which may regulate brain-specific gene transcriptions (reviewed currently by Flavell and Greenberg, 2008; Liu et al., 2008; Neary and Zimmermann, 2009), but those identified so far control steps in the proliferation, migration and differentiation of neurons, and some elements of morphogenesis. Despite the numerous GPCRs active in the CNS, a GPCR which regulates gene transcriptions for transmitter/receptor systems, as here, is truly exceptional. It should encourage a search for other GPCRs with similar dual roles in the brain.

Materials and Methods

Materials. Sprague-Dawley rats, from the Animal Care Facility of the University were used. All procedures with these were conducted under the Guidelines for the use and care of laboratory animals in research, of the Animal Research Panel of The Hong Kong University of Science and Technology. Cell culture media were from Invitrogen (Invitrogen Technologies, Carlsbad, CA). P2Y receptor agonists and antagonists were the purest grades available, from Tocris (Bristol, UK) or from Sigma (St. Louis, MO). Apyrase (Grade VII) and phorbol ester (TPA) were from Sigma. To ensure the purity of nucleotides, 2-MeSADP

and ADP stock solutions (1 mM) were pre-incubated with hexokinase/glucose (Roche, Mannheim, Germany), while ATP stock solution (1 mM) was pre-treated with creatine phosphokinase/creatine phosphate, all as described and validated elsewhere (Choi et al, 2001a).

Cell culture and drug treatment. Cultured cortical neurons and human embryonic kidney (HEK) 293T cells were prepared and cultured as described in Xie et al. (2009). In studies on mRNA and protein responses to drug treatments, cultured cortical neurons at 15 DIV were firstly treated with apyrase (1 U/ml, 1 h) to remove nucleotides in the culture medium, followed by washing once with neurobasal medium. To avoid interference due to the spontaneous neuronal activity in such cells, fresh medium containing tetrodotoxin (TTX, 100 nM, Sigma) was added to the culture and incubated for 3 hours, to obtain a stable baseline. Each drug was then added in fresh TTX-containing culture medium. Antagonists were present for at least 30 min prior to an agonist addition, and then throughout the treatment. In phosphorylation studies, the cultures were incubated in neuronbasal medium only for 3 h before the application of reagents.

mRNA analyses. Total cDNA from cortex of adult rats or from cultured cortical neurons was prepared as described in Xie et al. (2009). For qualitative detection of P2Y₁R mRNA, total cDNA from the cortex (500 ng) or cultured cortical neurons (200 ng) was amplified in each PCR assay with Taq DNA polymerase by the P2Y₁R primers, 5'-CCT

MOL #66506

GCG AAG TTA TTT CAT CTA-3' (forward) and 5'-GTT GAG ACT TGC TAG ACC TCT-3' (reverse). Control samples using rat genomic DNA (5 ng/reaction) *or* RNAs (50 ng/reaction for cortex and 200 ng/reaction for cultured cortical neurons) lacking reverse transcriptase were processed in parallel.

Real-time quantitative PCR. Real-time PCR was performed using SYBR green master mix and Rox reference dye according to the Roche instructions. The primers were: 5'-CTG GGG TGC GGA TCG GTG TAC CCC-3' (forward) and 5'-TCA CAG GTC TGA GCA GCG TTC CTG-3' (reverse) for the AChE_T variant, 5'-AAC GGA TTT GGC CGT ATT GG-3' (forward) and 5'-CTT CCC GTT CAG CTC TGG G-3' (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The SYBR green signal was detected on the Mx3000p multiplex quantitative PCR platform (Stratagene, La Jolla, CA). The transcript expression levels were measured using the $\Delta\Delta C_t$ value method (Xie et al., 2009), where values were normalized by the internal control GAPDH in the same sample. PCR products were analyzed as above, plus melting curve analysis to confirm the specific amplification.

cDNA plasmids, reporter gene constructs and transfection. Rat P2Y₁R cDNA was subcloned into the pCMV-Tag-3 vector (Stratagene) to produce N-terminal-Myc-P2Y₁R or into the pcDNA4 vector (Invitrogen) for tagging with N-terminal hemagglutinin-peptide (HA) by a standard PCR method using *Pfx* (Invitrogen). Each was confirmed by DNA

sequencing. Carboxyl-terminal truncation was made using a reverse primer flanking 985-1002 bp in rat P2Y₁R cDNA to form P2Y₁R_{ΔC-term} (Choi et al., 2008). Mutations in the carboxyl-tail, changing DTSL to DYSR, were made using the reverse primer 5'-AA CTC GAG TCA CCT ACT GTA GTC TCC-3' to form the P2Y₁R_{DYSR} construct (underlined: XhoI site). The human AChE promoter-reporter and Raf constructs, pAChE-Luc, pAChE_{ΔE1k-1 [1]}-Luc, pAChE_{ΔE1k-1 [3]}-Luc, pAChE_{ΔE1k-1 [1,3]}-Luc, Raf_{WT} and Raf_{CAAX} were as described in Choi et al. (2003). Cultured cortical neurons cultured for 3 days and 14 days, for differentiation and drug treatment studies respectively, were transfected transiently with the purified plasmids (0.5 μg per well in 24-well plates) by LipofectAMINE 2000-CD (Invitrogen). The transfection efficiency in the neurons was about ~5%. HEK 293T cells were transfected with calcium phosphate (Choi et al., 2003).

Immunoblotting and phosphorylation studies. The cultured neurons were collected in lysis buffer (100 μl per well of 6-well plate) containing 150 mM NaCl, 10 mM HEPES pH 7.5, 0.5% Triton X-100, 5 mM EGTA, 5 mM EDTA, 1 mg/ml bacitracin, 1 μg/ml leupeptin, 1 μg/ml aprotinin. Phosphorylation analysis and collection of the lysates were as in Siow et al. (2002). The final lysate supernatant was loaded on gels at 10 μl per lane. The proteins were separated on 8% or 12% SDS-polyacrylamide gels and electroblotted onto nitrocellulose filters for 16 h. Western blot analyses were carried out as described in Siow et al. (2002), the following commercial antibodies were used: anti-AChE antibody (1:2000, BD

Biosciences, Franklin Lakes, NJ), anti-P2Y₁R antibody (1:1000, Alomone, see next section), anti-NR1 antibody (1:5000, BD Biosciences), anti-NR2A antibody (1:1000, Millipore, Billerica, MA), anti-choline acetyltransferase (ChAT) antibody (1:2000, Millipore), anti-muscarinic acetylcholine receptor M1 (M1 mAChR) antibody (1:1000, Sigma), anti-PSD-95 antibody (1:5000, Millipore), anti-neurofilament (NF)-200 antibody (1:2000, Sigma), Cy3-conjugated anti-glial fibrillary acidic protein (GFAP) antibody (1:5,000, Sigma), anti-microtubule-associated protein 2 (MAP-2, Sigma) antibody (1:5,000, Sigma), anti-GAPDH and anti- α -tubulin antibodies (1:10,000, Abcam), anti-phospho ERK antibody and anti-ERK antibody (1:5000, Cell Signaling Technology, Danvers, MA). The immunoreactive bands were visualized by chemiluminescence with the ECL protocol (GE Healthcare, Piscataway, NJ). The labeling intensities of the protein band from the control and from the agonist-stimulated samples, run in the same gel, were compared by densitometry within the range of a calibrated density/response curve. α -Tubulin or GAPDH, as shown, were included as visual loading controls and used to normalize the densities before plotting.

Immuno-cytofluorescent staining. Cultured cortical neurons or cDNA-transfected HEK 293T cells were grown on glass cover-slips. They were fixed and stained as described previously (Tung et al., 2004). The following commercial antibodies were used in this study: anti-P2Y₁R (1:100; APR-009, Alomone Labs, Israel, to an epitope in the third intracellular loop of P2Y₁R); anti-AChE (1:500, BD Biosciences); Cy3-conjugated anti-GFAP (1:2000,

Sigma); anti-MAP-2 (1:500, Sigma); goat anti-neurogranin (1:100, Santa Cruz Biotechnology, Santa Cruz, CA); anti-PSD-95 (1:500, Millipore); or anti-SV48 (1:500, Xie et al., 2009). The cells were washed and stained with the corresponding Alexa 488- or 555-conjugated secondary antibodies (Invitrogen) plus nuclear stain TO-PRO-3 (1:500, Invitrogen), and then dehydrated serially in ethanol and mounted with fluorescence mounting medium (Dako, Carpinteria, CA). Confocal fluorescence microscopy (Leica, DMIRE2) was used with excitation (Ex) 488 nm/emission (Em) 500-535 nm (green), Ex 543/Em 560-615 nm (red); for nuclear staining, Ex 640/Em 660-750 nm for TO-PRO-3 or Ex 405/Em 465 for DAPI (both blue pseudo-color).

For co-localization of PSD-95 and P2Y₁Rs, PSD-95-GFP cDNA (Stratagene) was co-transfected with Myc-P2Y₁R_{ΔC-term} or Myc-P2Y₁R_{DYSR} cDNAs in HEK 293T cells. After transfection (48 h), cultures were and incubated with Cy3-conjugated anti-Myc antibody (1:500, Invitrogen) in blocking solution to recognize the membrane receptors only. The cultures were washed, mounted and analyzed by confocal microscopy as above.

Fluorometric measurement of Ca²⁺ mobilization. Cortical neurons on cover-slips mounted at the base of the reaction chamber were loaded with 2 μM Fluo-4-AM (Invitrogen) in 100 μl Hanks' balanced salt solution buffered with 20 mM of HEPES, pH 7.5 (HBSS), for 1 h at 37°C. Following 3 x 5 min washes with HBSS, serial images were captured in Leica confocal microscopy at Ex 488/Em 500-550 nm, and analyzed by Image J software to

MOL #66506

measure the change in intracellular Ca^{2+} . To determine the mean response in the cell population, cortical neurons (at 15 DIV) in black-walled, clear-bottomed 96-well culture plates at confluency were labeled at 37°C as above, followed by medium replacement with 150 μl HBSS. For each assay, the agonist was added in 50 μl HBSS. To obtain a stable baseline reading, tetrodotoxin (TTX, 100 nM, Sigma) was added to the buffer. Changes in fluorescence were measured in a FlexStation II plate-reader (Molecular Devices, Sunnyvale, CA) at Ex 488/Em 520 nm. Data were expressed as changes in fluorescent intensity relative to the basal (untreated) value and analyzed with GraphPad Prism 3.

Immunoprecipitation and pull-down assays. Lysates from cultured cortical neurons or transfected HEK 293T cells in 100-mm plates were prepared in modified RIPA buffer (20 mM sodium phosphate, 1% Triton X-100, 1 $\mu\text{g}/\text{ml}$ pepstatin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{ml}$ aprotinin, 2 mM sodium orthovanadate and 50 mM sodium fluoride). The lysates (~500 μg protein per lysate) were incubated with protein G-agarose (Roche) suspension (50% slurry, 50 μl) for 2 h at 4°C. The supernatant was then incubated with specific antibodies (~4 μg) and gently rotated at 4°C overnight. After that, another 50 μl protein G-agarose suspension was added and gently rotated for 5 h at 4°C. The collected beads were washed 3 times with modified RIPA buffer and then with 50 mM Tris-HCl, pH 8.0. Finally, the beads were re-suspended in SDS sample buffer (25 μl) and heated at 100°C, 5 min. The extracts were analyzed by SDS-PAGE and immunoblotting.

Other assays. Protein concentration and AChE enzymatic activity determinations, and the separation of AChE molecular forms on sucrose density gradient, were as in Xie et al. (2009). The validation of the G₄ AChE isoform by anti-PRiMA antibody was also as in Xie et al. (2009). Luciferase assay was carried out on the cultures of cells expressing the luciferase constructs noted above, as described in Siow et al. (2002).

Data analysis. Gel documentation and relative quantification were performed with the IS1000 Digital Imaging System. For the co-localization ratios, twenty neurons were randomly selected from each double-immuno stained coverslip on the basis of healthy morphology using phase-contrast. Then, the numbers of immuno-positive puncta were counted per 50- μ m dendrite length. Only puncta larger than 10 pixels were counted to avoid background staining. The co-localization ratio (%) of each two immunopositive puncta was calculated: [number of puncta double-immune stained per number of total puncta immune stained with one of the two antibodies] x 100%. On average, 80 puncta were analyzed per neuron.

All statistical analyses were by two-tailed, unpaired, one-way ANOVA. Data are plotted as mean \pm SEM (except where the bars fall within a symbol), for $n = 4$ independent experiments (unless stated otherwise), each using triplicate samples. Other Figures are likewise representative of 4 or more replicates.

MOL #66506

Results

Expression and localization of P2Y₁ receptors in neurons of the cortex. P2Y₁R mRNA was found to be well expressed both in the intact adult rat cerebral cortex and in the cortex neurons in primary culture used in this study (Fig. 1A). We have used here primary cultures derived from developing rat cortex, in development up to 25 DIV, in conditions in which the neurons could be studied specifically, to investigate P2Y₁Rs thereon. Our untreated cultures contained neurons and many glia and showed strong reaction both for MAP-2 (a neuronal marker) and for the astrocyte marker GFAP, in immuno-cytofluorescent staining and in Western blotting (Fig. 1B). A schedule of treatment with the glial-suppressant Ara-C (see Materials and Methods) reduced the astrocyte content to a negligible level, as indicated by selective disappearance of anti-GFAP reactivity (Fig. 1B). In the conditions used, the neuron-rich cortical cultures are fully viable, as has been shown by others (Hilgenberg and Smith, 2007; Zhang et al., 2003). These were used (except where stated) in all of the analyses in culture here. In view of several reports (e.g. Yang et al., 2003) that the differentiation of brain neurons is retarded if cultured as here in the absence of astrocytes, due to the lack then of the astrocyte-derived activator D-serine, we tested the effect of including D-serine (10 μ M) in the neuronal cultures. The expression of three neuronal proteins studied here as reported below, AChE, ChAT and NR2A, was found to show no change in this medium in their

expression levels throughout the culture period, hence validating the culture conditions used here (data not shown).

In immuno-blots from such treated cortical neurons when probed with Alomone anti-P2Y₁R antibody (Fig. 1C), bands of proteins migrating at ~60 kDa and ~42 kDa were detected (specificity being shown by their elimination by the presence (Fig. 1D) of the corresponding peptide antigen). The product at ~42 kDa agrees with the predicted size of the P2Y₁R protein. The band at ~60 kDa was also seen by Moore et al. (2001) in immuno-blots (with a different anti-P2Y₁R antibody) derived from whole rat brain and is attributable to post-translational glycosylation. These blots allowed us to see that the relative expression of the P2Y₁R protein increased markedly during neuronal differentiation in culture, starting at 5 DIV. The expression was at a plateau after 20 days of culture (Fig. 1C). In parallel, the expressions of NF-200 at ~200 kDa and PSD-95 at ~95 kDa, both being differentiation markers for neurons, increased similarly then, again reaching a plateau near 20 DIV. The expression of the neuronal “housekeeping” α -tubulin gene was little changed from 10 DIV onwards, serving as an internal loading control (Fig. 1B).

To investigate distributions of P2Y₁Rs in the intact neurons their immuno-cytochemistry was required, but under the usual conditions for that technique the risk of non-specificity in some anti-GPCR antibodies is significant. We have shown (Tung et al., 2004) that in immuno-cytochemistry the above-noted commercial anti-P2Y₁R antibody

can give cross-reaction with some of the other P2YR subtypes, and that this can be blocked by applying it in medium containing relatively high BSA levels, maintained before and during the reaction. This medium was employed here. For certainty, the specificity for P2Y₁R of the antibody used in the immuno-blots and in immuno-cytochemistry was confirmed by a stringent criterion, i.e. showing identical results in a subsequent test comparison with a new anti-P2Y₁R antibody recently validated in testing on brain of P2Y₁R-knockout mice (shown in Supplemental Data S1).

It was thus shown in well-differentiated cortical neurons that the P2Y₁Rs are relatively strongly expressed on most of the dendrite-like neuritis, as well as on the cell bodies (data not shown). On the dendrites the P2Y₁Rs showed a punctate localization (Fig. 1E). Of the P2Y₁R puncta (on 20 neurons counted), $54.2\% \pm 2.9\%$ were localized at the postsynaptic densities (PSDs), as shown by their marker protein PSD-95, relative to the total PSD-95-expressing puncta on those dendrites. A similar overall association with PSD-95 was seen for P2Y₁Rs present at cell body membrane regions, but those, being less punctate, could not be quantitated accurately. In contrast, the receptor on the dendritic puncta showed less co-localization with a pre-synaptic marker, anti-synaptotagmin (SV48) antibody, i.e. $26.3\% \pm 2.1\%$ (on 20 neurons), relative to the total SV48-containing puncta (Fig. 1E). The difference in these values is significant at $p < 0.001$ and at this confocal resolution level the percentages show that the P2Y₁Rs on cortical neuron dendrites are mainly post-synaptic. The apparently

pre-synaptic minority would not be well resolved here from the astrocyte processes carrying P2Y₁Rs when those processes are closely-apposed to the excitatory synapses, as is common in the forebrain (Perea et al., 2009): i.e. there may be still fewer, if any, P2Y₁Rs on the neuronal pre-synaptic membranes. While these analyses were performed in confocal microscopy, further study at higher resolution was not made here since extensive quantitative electron-microscopic analysis became available of immunogold labeling of forebrain P2Y₁Rs by identical antibody (applied in a serum albumin-enriched medium similar to ours) in rat hippocampal sections (Tonazzini et al., 2007). That study has shown clearly the predominant localization of P2Y₁Rs at neuronal PSDs, fully consistent with our results on cortex cultures; it could show, further, that the neuronal P2Y₁Rs are associated with the glutamatergic class of synapses. Tonazzini et al. (2007) also found that the astrocyte cell membranes there showed the second-highest density of the P2Y₁R, in accord with its activity seen in functional studies on astrocytes in brain tissue (Zhang et al., 2003; Bowser and Khakh, 2004; Perea et al., 2009).

P2Y₁ receptors interact with PSD-95 by PDZ association. The carboxyl terminus of all known P2Y₁R sequences ends in the conserved motif D-T-S-L, a canonical binding motif for Class I PDZ domains (Bockaert et al, 2003). This tetrapeptide in P2Y₁R has been shown (Fam et al., 2005) to interact with a PDZ-domain from the scaffold protein NHERF-2 (Na⁺/H⁺ exchanger regulatory factor type 2). In CNS neurons, PSD-95 is a more

generally-occurring postsynaptic scaffold protein, and contains three PDZ domains as well as two of other types of binding domain (Kim and Sheng, 2004). We found using extracts of cultured cortical neurons, the native P2Y₁Rs could be co-immunoprecipitated with PSD-95 and *vice versa* (Fig. 2A). The NR2A subunit of N-methyl-D-aspartate (NMDA) receptors served as a positive control, due to its known association with PSD-95 (Kim and Sheng, 2004). Interestingly, the NR2A subunit could also be pulled down by anti-P2Y₁R antibody (Fig. 2A). These specific pull-down experiments show protein interaction in cortical neurons of PSD-95 with P2Y₁R, and also of NMDA receptors with P2Y₁Rs. Since the latter pair differs in containing no PDZ domains, we presume that each of those receptor types attaches directly to PSD-95 *in situ*.

The interaction of PSD-95 with P2Y₁R was further demonstrated in HEK 293T cells heterologously expressing both of those proteins. Considering the above-noted requirement for the C-terminal DTSL motif, cDNAs encoding one of three forms of the P2Y₁R were constructed: N-terminal-HA-tagged wild type P2Y₁R (HA-P2Y₁R), HA-tagged P2Y₁R with a mutated C-terminus (HA-P2Y₁R_{DYSR}) and HA-tagged P2Y₁R with the deletion of the cytoplasmic tail (HA-P2Y₁R_{Δc-term}) (Fig. 2B). These cDNAs were expressed to provide immuno-blots using (alone) each of the corresponding antibodies (Fig. 2B). These cells showed a band at ~95 kDa for PSD-95 and at ~42 kDa (lower gel, with anti-HA) for P2Y₁R, except for the shorter HA-P2Y₁R_{Δc-term} giving a lower band (~37 kDa). In the

MOL #66506

cDNA-transfected HEK 293T cells the P2Y₁R was co-immunoprecipitated with PSD-95 and *vice versa* (Fig. 2C). Hence the result was similar to the situation in the brain extract. However, the mutants, HA-P2Y₁R_{DYSR} and HA-P2Y₁R_{ΔC-term}, were unable to co-immunoprecipitate with PSD-95 (Fig. 2C). Thus, the P2Y₁R-PSD-95 association is mediated by the DTSL motif of the receptor.

To confirm the association of P2Y₁R with PSD-95 in intact cells, HEK 293T cells were co-transfected to express PSD-95 (carrying a green fluorescent protein (GFP) label downstream) plus one of the alternative P2Y₁R forms as in Fig. 2B. In this case the receptors were N-terminally tagged with the Myc peptide in place of the HA tag, and visualized by the red fluorescence of Cy3-labeled anti-Myc antibody (Fig. 3A). In all cases where P2Y₁R was expressed, it was seen as clearly localized at the cell membrane: its internal fraction (expected in growing cultures) was avoided in the imaging since the directly-labeled anti-Myc antibody used for it is to an N-terminal tag and no permeabilization was given here. In contrast, when the labeled PSD-95 was expressed alone it was seen to be present throughout the cytosol (Fig. 3A). However, in cells expressing both P2Y₁R and PSD-95, most of the PSD-95 became co-localized with membrane P2Y₁Rs (Fig. 3B, *lower panels*). In controls co-transfected to express P2Y₁R and free (unconjugated) GFP, GFP remained throughout the cytosol and nucleus (Fig. 3B, *upper panels*). Thus, the P2Y₁R is one of the partners of PSD-95 which facilitates its association with the cell membrane. However,

alteration at the C-terminus of P2Y₁R, both in the P2Y₁R_{DYSR} and P2Y₁R_{ΔC-term} constructs, removed this ability. Thus, in cells co-expressing PSD-95 with P2Y₁R_{DYSR} or with P2Y₁R_{ΔC-term}, PSD-95 was found in the cytosol and did not co-localize with the receptor, even though the mutant receptor was still maintained at the membrane (Fig. 3C, 3D, *lower panels*). In summary, the C-terminus of the P2Y₁R interacts at its DTSL motif with PSD-95, and formation of this complex can recruit intracellular PSD-95 to the cell membrane. A mechanism for such PSD-95 migration is known (Kim and Sheng, 2004), since another of its PDZ domains binds strongly to the C-terminus of a kinesin molecular motor to stimulate translocation of its complexes along micro-tubule tracks to the cell membrane region.

P2Y₁ receptor-mediated intracellular Ca²⁺ mobilization in neurons. The P2Y₁R is well-established to be in the family of G_{q/11}-linked receptors and thus is expected to signal upon agonist activation primarily via phospholipase-C, IP₃ and mobilization of Ca²⁺ from intracellular stores (reviewed in Abbrachio et al., 2006). With rat cortex mixed-cell cultures, Bennett et al. (2003) found that only 9-15% of the neurons mobilized Ca²⁺ in response to P2Y₁R agonists (but all glia did so), in testing at an earlier stage than ours (E14 cortex, cultured only for 8 days, and with ~50% glia present). Therefore, our cortex cultures, being more mature and with glial suppression, were compared here in this response.

We have demonstrated previously in the case of cultured myotubes (Choi et al., 2003; Tung et al., 2004) that activation by adenosine tri- or di-phosphates of P2Y₁R or P2Y₂ R

mobilizes intracellular Ca^{2+} and, further, this leads to an increase in the phosphorylation of the ERK1/2 kinases. Earlier work on transfected cell (Sellers et al., 2001) has indicated that this pathway for $\text{P2Y}_1\text{R}$ involves the MEK1 kinase and phosphatidylinositol-3-kinase. Here, cortical neurons in culture were pre-loaded with the Ca^{2+} indicator Fluo-4, and the mobilization of intracellular Ca^{2+} upon agonist stimulation was monitored in real time with the confocal microscope. The intracellular Ca^{2+} sharply increased across the neuron in less than 20 s after the application of 2-MeSADP, a highly potent $\text{P2Y}_1\text{R}$ agonist (Fig. 4A, B). A control application of buffer alone induced no Ca^{2+} mobilization (data not shown). In quantitative measurements, all of the neurons tested gave this response. The mean EC_{50} is 71.3 ± 2.8 nM (Fig. 4C). The 2-MeSADP-induced intracellular Ca^{2+} accumulation could be blocked (Fig. 4D) by co-incubation with the antagonists MRS2179 ($\text{P2Y}_1\text{R}$ -specific: Camaioni et al., 1998) or A3P5P (adenosine-3', 5'-bisphosphate, $\text{P2Y}_1\text{R}$ -selective) or with the general P2 antagonist suramin. The activation of endogenous $\text{P2Y}_1\text{Rs}$ in cultured cortical neurons by 3 agonists (2-MeSADP, ATP and ADP) led to the strong phosphorylation by each agonist of the MAP-kinase ERK1 (~44 kDa) and (at a low level) ERK2 (~42 kDa). A phorbol ester activator of protein kinase C (PKC), TPA, served as a positive control, inducing a strong (~4-fold basal) phosphorylation of ERK1/2 (Fig. 4E, upper panel). The ERK1 and ERK2 total protein content remained invariant. The scanned data showed that the phosphorylation of ERK1/2 evoked by the more selective $\text{P2Y}_1\text{R}$ agonist 2-MeSADP is strong and highly

significant (Fig. 4E, *lower panel*). Owing to the evidence for interaction of PSD-95 and P2Y₁R at the plasma membrane (Fig.3), it was interesting to know whether PSD-95 would affect the receptor signaling response in terms of Ca²⁺ mobilization and ERK phosphorylation. Therefore, a differential expression test in HEK293T cells was employed for that purpose. The results indicated that there was no significant difference between P2Y₁R alone and P2Y₁R plus PSD-95 in terms of both Ca²⁺ mobilization and ERK phosphorylation (Supplemental Data S2). These results suggest that the role of PSD-95 here is to serve as a scaffold protein to concentrate P2Y₁R at sites on the plasma membrane rather than to regulate the receptor signaling.

It can be shown that this finding of functional P2Y₁Rs on the embryonic cortical neurons in culture here is representative of native embryonic cortex *in situ* and also of differentiated neurons in the postnatal forebrain. Similar results were found in a recent study of P2Y₁Rs on acute slices of day-E16 mouse neocortex (Liu et al., 2008). There, abundant identified post-mitotic neurons responded to fast-applied ATP with strong Ca²⁺ transients, which could be fully blocked by MRS2179 or suramin as was seen here. Further, the responsive receptors on native cortical neurons were confirmed as P2Y₁Rs by the suppression of the transients after electroporation of P2Y₁R short-hairpin (sh)RNA into those cells. Also, in postnatal development strong electrophysiological activity identified with P2Y₁Rs has been shown on pyramidal cells and interneurons of the hippocampus, either in 2-week culture

MOL #66506

from rat postnatal day P3 (Filippov et al., 2006) or in acute slices from P7-15 (Kawamura et al., 2004). The latter case was confirmed by complete loss of those responses in transgenic P2Y₁R deleted mice. Further, from rats aged 6 weeks, single cell PCR analysis of pyramidal neurons in hippocampal acute slices has confirmed that strong expression of P2Y₁Rs in maintained thereon (Rodriguez et al., 2005).

Release of ATP onto cortical neurons. Although functional P2YRs were shown thus to be available here, the extents of endogenous release of ATP onto cultures of brain neurons and of glia have not been previously reported. The concentration of ambient ATP in the bulk phase above the monolayer of cortical neurons in our conditions was measured and found to be low, being limited by the high surface ecto-nucleotidase activity, but it was increased ~6-fold when the cultures contained freely growing glial cells (Supplemental Data S3). In agreement, we have found a similar endogeneous release of glial ATP from the NG108-15 neuroblastoma/glioma cells cultured alone (Ling et al., 2005). For comparison, activation of P2YRs on cultured cortical astrocytes strongly mobilizes their intracellular Ca²⁺ to release glutamate, which when occurring *in situ* would activate adjacent neuronal NMDA receptors (Lee et al., 2007). The actual local concentration of agonists at the P2Y₁Rs at the surface of the cultured cortical neurons is predicted to be, in reality much higher than those measured in the bulk medium above them and to be sufficient to activate them (for details, see

Supplemental Data S3). The relationship of these findings to the treatments which we make with ATP and its derivatives is considered in the Discussion (second paragraph).

ATP induces neuronal gene expressions. At vertebrate neuromuscular junctions, activation of P2Y₁Rs leads to a marked increase in transcriptional activity at the *ACHE* gene (encoding the catalytic subunit of AChE), with some of the elements responsible for that gene activation identified (Choi et al., 2003). This suggests a potential role of P2Y₁R in regulating the *ACHE* gene in brain neurons. Hence, neuronal AChE was the first synaptic protein analyzed thus in this study. Different forms of AChE (based upon the same catalytic subunit and *ACHE* gene) have been identified at various locations, e.g. globular and collagen-tailed forms (Xie et al., 2007), and these were distinguished here.

The enzymatic activity of AChE increased during the neuronal differentiation process, and reached a maximum activity after 20 DIV (Fig. 5A). The time-course of expression of the AChE catalytic subunit protein at ~68 kDa, revealed by Western blotting and of its mRNA measured by real-time PCR, was in accordance with the increase of activity (Fig. 5A). In cultured cortical neurons, a globular form of AChE with a sedimentation constant of 11S was the most abundant form identified (Fig. 5B). This AChE type corresponds to a known 11S form, G₄, a tetrameric enzyme linked to Proline-Rich Membrane Anchor (PRiMA: Perrier et al., 2002), an anchoring transmembrane 20 kDa protein. This identification was supported by

observing that the 11S form of AChE was selectively depleted by reaction with anti-PRiMA antibody (Fig. 5B).

The localization of AChE in cultured cortical neurons was revealed using an AChE-specific antibody. By using immuno-cytofluorescent staining, AChE showed a punctate localization on the dendrites dendrites, which was similar to that revealed for P2Y₁R. When co-stained with PSD-95 antibody, 51.9% \pm 5.0% of these AChE puncta (counted on 20 neurons, and relative to the total number of PSD-95 puncta) were found to be co-localized with PSD-95 (Fig. 5C). The results therefore suggest a mainly postsynaptic localization of AChE in cortical neurons.

A potential regulatory function of the P2Y₁R, to induce the expression of the AChE catalytic subunit (as noted above for muscle), was explored in cultured cortical neurons. After the addition to the culture medium of 2-MeSADP or ATP at 50 μ M for 24 h, the expression of transcript encoding AChE rose to a maximum of 4.5-fold compared to the level in the ligand-free control culture (Fig. 6A). Addition of TPA (10 nM), activating PKC, served as a positive control, increasing AChE transcript expression to ~3-fold. The content of AChE catalytic subunit protein (~68 kDa) was increased to a maximum of ~3-fold when P2Y₁R agonists (2-MeSADP, ADP and ATP) were applied. The extents (Fig. 6A, B) are consistent with their known relative potencies (Abbracchio et al., 2006) at mammalian P2Y₁R. This

P2Y₁R agonist-induced AChE expression was blocked by co-incubation with MRS2179, a P2Y₁R receptor-specific antagonist (Fig. 6B).

The promoter element of the human *ACHE* gene has been cloned (Ben-Aziz et al., 1993) and well characterized (Siow et al., 2002; Choi et al., 2003; Gao et al., 2009). The DNA (~2.2 kb) encompassing the human *ACHE* promoter was subcloned into pGL3 vector immediately upstream of a *luciferase* gene, designated as pAChE-Luc (Choi et al., 2001b; Siow et al., 2002). In cortical neurons transfected by pAChE-Luc, the promoter activity increased with the period of culture (Fig. 6C); this increase was parallel to that seen for the expression of endogenous AChE protein in these cells (Fig. 5A). In transiently-transfected cortical neurons, application of 2-MeSADP induced *ACHE*-promoter-driven luciferase activity, by ~2.7-fold. Again, co-incubation with MRS2179 blocked this P2Y₁R response (Fig. 6D). This initial stage of promoter activation is, not surprisingly, more sensitive to such regulation than the downstream stages of mRNA and protein production (Fig. 6A, B), so that even the weaker agonist ATP can then (Fig. 6D) evoke the maximum effect seen.

The downstream activation of this P2Y₁R-induced gene expression was also probed. Thus, a PKC activator, TPA, potently induced the promoter activity in a dose-dependent manner: at 5 nM TPA the induction was 4-fold that in untreated cultures (Fig. 6E). Testing a known pathway via the kinase Raf-1, two cDNA constructs were used: wild type (Raf_{WT}) and a constitutively-active, membrane-targeted mutant (Raf_{CAAX}; Schonwasser et al., 1998).

When these cDNAs were co-transfected with pAChE-Luc into cultured neurons, they each produced an increase in *ACHE* gene promoter activity. A greater and robust induction, to ~6-fold, was revealed in the Raf_{CAAX}-expressed neurons (Fig. 6E), suggesting a Raf signaling pathway is involved in regulating the transcriptional activity of the *ACHE* gene promoter.

Elk-1 is one of the transcription factors that can be phosphorylated through Raf/MEK/ERK pathway; this phosphorylation of Elk-1 subsequently activates the gene transcription of the *ACHE* gene (Choi et al., 2003). A search in the promoter region of the human *ACHE* gene sequence for potential binding sequences for Elk-1 had revealed two functional sites downstream of the 5'-untranslated exon 1; these 2 sites have been demonstrated to be responsive to P2Y₁ receptor activation in muscle (Choi et al., 2003). They are located in the first intron, at -1431 to -1412 bp and at -1102 to -1083 bp upstream of the ATG start site, designated as Elk-1[1] and Elk-1[3] respectively, as shown in Fig. 6F. Association of these Elk-1 binding sites in the *ACHE* gene promoter with P2Y₁R activation was further demonstrated here. The ~2.2 kb upstream sequence of human *ACHE* gene which includes the promoter region was mutated at those Elk-1[1] and Elk-1[3] elements, singly or together, to change six consecutive nucleotides in each. These mutant promoters were then tagged downstream with the luciferase reporter, to give the constructs: pAChE_{ΔElk[1]}-Luc, pAChE_{ΔElk[3]}-Luc and pAChE_{ΔElk[1,3]}-Luc (Fig. 6F). When these DNA constructs were

transfected into the cultured neurons, the mutated constructs were found to have lost all of the nucleotide-induced activation of the *ACHE* promoter activity (Fig.6F, *right panel*).

To investigate whether this P2Y₁R-mediated gene regulation extends to other components of neuronal signaling, we selected for study some genes involved in the NMDA-glutamatergic and cholinergic systems. By immuno-blot analysis the native expression was demonstrated in the cultured neurons of at least 1 subunit of the NMDA receptor (NR2A at ~170 kDa), of the acetylcholine-synthesis enzyme ChAT at ~65 kDa and of the muscarinic acetylcholine receptor (mAChR) subtype M1 at ~50 kDa. The protein expressions of these components increased during neuronal differentiation (Fig. 7A). By comparing the differentiated and undifferentiated neurons, an increase to 3 to 7 fold was revealed for them (Fig. 7A). Upon treatment of the neuronal cultures with 2-MeSADP (50 μ M for 24 h), the gene expressions of NR2A and ChAT were induced significantly, and co-incubation with the P2Y₁-specific antagonist MRS2179 blocked this response (Fig. 7B). The greatest effect was seen for ChAT, up to ~3-fold. No significant effects were seen at the muscarinic AChR M1 subunit gene (Fig.7B). These data, taken with the preceding results, indicate that activation of the native P2Y₁Rs on neurons of the cortex can differentially regulate the gene expression of some of the effector components of glutamatergic and cholinergic CNS synapses.

MOL #66506

Discussion

Expression of P2Y₁R (mRNA and protein) was clearly seen on cortex neurons, in adult brain and in culture (Fig. 1). This extends the previous findings on P2Y₁R mRNA in the cortex. Thus, Moore et al. (2001) found it is strongly expressed in total extracts of the adult human cortex and Bennett et al. (2003) showed that it is abundant in extracts of E14 rat cortex and in mixed-cell cultures derived from it, and in glia cultured alone. Specifically, in the multi-process-bearing astrocytes freshly isolated from the rat cortex or hippocampus 10-30% of the cells have been found to contain P2Y₁R mRNA and to show P2Y₁R functional responses, with this proportion rising to ~ 90% of astrocytes after 2 weeks in culture (Zhu and Kimelberg, 2001). P2Y₂R mRNA was absent initially. Webb et al. (1998) mapped P2Y₁R mRNA by its quantitative *in situ* hybridization in the chick brain: it is strongly expressed in the avian precursor of the mammalian cortex as well as in the hippocampus, cerebellum and the striatal complex, and has a strong presence there in the neurons. These lines of evidence raised our interest in an investigation of the functional role of P2Y₁R in the brain. We had previously shown the presence of P2Y₁R at skeletal nerve-muscle synapses and its role there in the control of gene expression of cholinergic-effector proteins (see Introduction). This is extended here to the very different neuron-neuron synapses of the brain and to non-cholinergic transmission.

A recent consensus has developed that ATP is constantly released onto brain neurons, in part from other neurons but largely from astrocytes (e.g. Zhang et al., 2003; Bowser and Khakh, 2004; Perea et al., 2009). Most synapses of forebrain neurons *in situ* are tightly enwrapped by astrocyte processes, with most astrocytic ATP release focused onto synapses (Perea et al., 2009). In our cultures ATP release was indeed greatly increased by glial presence (Supplemental Data S3). Most released ATP is rapidly hydrolyzed at neuronal surfaces, but mainly to ADP rather than adenosine by the triphosphate-preferring nucleoside-triphosphate-diphosphohydrolase-2 (NTPDase2) which is the main ecto-nucleotidase thereon (Neary and Zimmermann, 2009). ADP has a much higher agonist potency than ATP at mammalian P2Y₁Rs (Simon et al., 2001; Abbrachio et al., 2006), hence its action is significant at sub-micromolar levels: levels of ADP plus ATP predicted in the unstirred surface layer (Joseph et al., 2003) from constant ATP release, further stimulated via glutamate released in brain activity (Zhang et al., 2003), would be ample to elicit P2Y₁R responses on those neurons *in situ* (Supplemental Data S3).

Signaling steps in the ATP-induced *ACHE* gene expressions. The pathway of gene regulation by P2Y₁R in cortical neurons was explored for one target, the *ACHE* gene. AChE is a multi-functional protein well-known for its classical role as a hydrolytic enzyme for synaptic acetylcholine, as well as its non-classical roles such as as an adhesion protein to

promote cell-to-cell recognition or to induce signal transduction events under different physiological conditions. Thus, several neuronal binding partners of AChE are known, including beta-amyloid (Alvarez et al., 1997), RACK1 (Birikh et al., 2003), laminin-1 (Paraoanu and Layer, 2004) and $\alpha 7$ -nAChR (Greenfield et al., 2007). Hence the finding of a regulation of the brain AChE gene activation via a transmitter has a wider significance.

Activation of P2Y₁R in cortical neurons induced mobilization of intracellular Ca²⁺ and ERK1/2 expression, as is known for P2Y₁Rs in recombinant expression (Sellers et al., 2001) and similarly when endogenous, as in muscle cells (Choi et al., 2003). This activation induced increases in AChE mRNA and protein and in *ACHE* gene promoter activity, involving protein kinase C and Raf-1. This leads to phosphorylation of Elk-1 and its action at a responsive element within the *ACHE* gene promoter, already known (Choi et al., 2003) to act thus in muscle.

Regarding the P2Y₁R-induced AChE expression, the AChE enzymatic activity (attributed to the AChE_T variant) remained unchanged (data not shown). That phenomenon occurred likewise in our previous studies in muscle (Choi et al., 2001a; 2003) and in other findings (Rotundo, 2003), where the AChE_T protein existed as both active and inactive pools. Interestingly, such an observation in the nervous system was different from that in the immune system; the change of mRNA/protein expression of AChE_R variant corresponded to

the change of AChE activity in splenocytes (Shaked et al., 2009), suggesting that the control of enzymatic activity would be isoform-specific.

The predominant form (11S) of AChE in mammalian brain is known to differ from that in skeletal muscle. The catalytic subunit (AChE_T) is the same in both but in brain it assembles as a tetramer anchored at the neuronal membrane through another (PRiMA) subunit, whereas muscle subunits assemble differently as multimers around a collagen-tail subunit and insert thereby into the extracellular matrix (Perrier et al., 2002). It was hitherto unknown whether the regulation of *ACHE* gene expression in brain neurons would operate as seen (Choi et al., 2003) in muscle, but we find here that its P2Y₁R-initiated pathway is common to both. However, a clear distinction in this between the two tissues is known at the promoter level (Camp et al., 2005): a deletion in transgenic mice of a 255-bp region of *ACHE* intron-1 suppressed its expression in muscle but not in brain. That region contains an N-box and an E-box motif which exert *ACHE* gene promoter activity in muscle by binding other trophic factors (Schaeffer et al., 2001), but those motifs are distant from the Elk-1 sites involved here. Hence, the AChE promoter responsive to Elk-1 via nucleotide/P2Y₁R activation is distinct, both by its location in the gene and its operation in both muscle and brain expression. Such distinguishing characteristics suggest that this nucleotide activation plays a significant role in brain neuronal function.

Neuronal P2Y₁ receptors are in a postsynaptic micro-domain. We found that in well-differentiated neuronal cultures the major fraction of the P2Y₁Rs is located either in the cell body membrane region or at punctate sites (developing spines) on the dendrites, strongly co-localized there with the post-synaptic protein PSD-95. As we noted, this agrees with electron-microscopic evidence (Tonazzini et al., 2007) that P2Y₁Rs in hippocampal sections are predominantly localized at the PSD. Co-anchorage of different receptors plus their effectors in microdomains via such scaffolds is currently seen as integrating much G_{q/11}-based signaling (Bockaert et al., 2003; Delmas and Brown, 2004; Kim and Sheng, 2004). This is now extended to P2Y₁R, as concluded from that co-localization plus several lines of additional evidence (Figs. 1-3). Firstly, P2Y₁Rs were co-immunoprecipitated from lysates of native cortex with PSD-95 (and *vice versa*) or with NR2A subunits from cortical neuron extracts, by antibody to any one of those three proteins; NR2A is already known (Kim and Sheng, 2004) to bind to PSD-95. Secondly, after PSD-95 was expressed in HEK 293T cells it was cytosolic but could largely be recruited to the cell membrane by co-expressed P2Y₁R. Thirdly, since such PSD-95 attachments occur at one of its PDZ domains through the C-terminal tetrapeptide of each ligand protein (Kim and Sheng, 2004), which in P2Y₁R is DTSL, we mutated or deleted that motif. This abolished the co-immunoprecipitation of PSD-95 and P2Y₁R, and also their membrane co-localization.

Another mode of anchoring P2Y₁Rs is known, via a PDZ-domain in NHERF-2 (Fam et al., 2005) and we have found that P2Y₁R in cells can dimerise by binding thus to NHERF-2 (Choi et al., 2008). NHERF-2 is present post-synaptically in forebrain neurons *in situ*, as shown in the mouse cortex (Paquet et al., 2006), and we found P2Y₁R also located there. NHERF-2 is presumed to contribute to the organization of P2Y₁Rs at the membrane. However, contrary to the similar conclusion drawn here for PSD-95, Fam et al. (2005) observed no binding of P2Y₁Rs to PSD-95. This was based upon screening a proteomic array of PDZ-domains against an overlay of a P2Y₁R C-terminal 50-amino-acid peptide, fused to glutathione-S-transferase for detection. The PDZ-domain isolated from the PSD-95 sequence did not bind to that region of the P2Y₁R in this system. Positive results from this screen have identified various PDZ-domain/receptor interactions, including NHERF-2/P2Y₁R (Fam et al., 2005; Paquet et al., 2006). However, we considered whether another interaction might be made there for certain pairs such as PSD-95/P2Y₁R. Thus, firstly, the full-length PSD-95 is known from crystallographic and other evidence to require a specific folding for a PDZ-domain to bind a GPCR partner held within the PSD-95 tertiary structure (Kim and Sheng, 2004, and references therein) and this may not always be attainable in an isolated PDZ-domain/fusion protein screen. Secondly, in exceptional cases PDZ complexing may require a non-tail additional binding site in the GPCR, as with the mGluR1/tamalin scaffold PDZ-based pairing: there such screening gave negative results (Paquet et al., 2006), but other

methods (Hirose et al., 2006) show that a second binding site also acts, located between 89 and 179 amino-acids from the mGluR1 C-terminus (S-S-T-L). The co-immunoprecipitation and DTSL-dependence tests which we applied (Figs. 2, 3) to demonstrate this for P2Y₁R/PSD-95 had likewise given positive results when employed (Fam et al., 2005) in confirming the P2Y₁R/NHERF-2 attachment in heterologous co-expression.

We also considered the possibility that the P2Y₁R/PSD-95 interaction shown might be indirect, through bridging by NHERF-2 binding to both, using its two NHERF-2 PDZ-domains. Thus, some PDZ-domain-containing scaffolding proteins can indeed link to another family member, to form hetero-multimers (Lau and Hall, 2001); however, in that study (using co-immunoprecipitation of the full-length proteins), while NHERF-2 showed linkage to NHERF-1 or to itself, no NHERF-2 binding was found to PSD-95 nor to any of several other PDZ proteins tested. Other potentially-bridging neuronal scaffolds not tested here can also be disregarded for this, since we additionally showed that the P2Y₁R/PSD-95 interaction is strong in transfected HEK cells. (For NHERF-2 itself, while also endogenous in our HEK cells, it is there only at a low level (Choi et al., 2008) and could not account for these effects (Figs. 2 and 3) on the much larger amounts of interacting P2Y₁R and PSD-95 heterologously expressed there). Hence, direct P2Y₁R/PSD-95 interaction remains the most likely explanation of our findings.

Relationships of P2Y₁ receptors to neuronal functions. Activation of forebrain P2Y₁Rs is known electrophysiologically to modulate neuron excitability (Bowser and Khakh, 2004; Perea et al., 2009), and to inhibit the M-current K⁺-channel to increase by ten-fold the firing rate of identified pyramidal neurons (Filippov et al., 2006). The modulation of excitability was proposed, in the studies cited, to arise from a relationship of synaptic P2Y₁Rs to NMDA receptors. P2Y₁Rs localized at a microdomain based on PSD-95 and NHERF-2, as deduced here, can provide the basis for this and also for the P2Y₁R signaling cascade to selective gene transcriptions.

While most of the activating ATP/ADP derives from glia (see above), neuronal ATP/P2Y₁R Ca²⁺-mobilization was also seen here with glia absent, plus longer-term gene-regulation of signaling components. We suggest this behavior contributes to the finding that blocking P2Y₁R by its antagonists or short-hairpin RNA (shRNA) impairs the proliferation and migration of the intermediate neuronal progenitors (INPs) in the developing mouse cortex (Liu et al., 2008). In addition, the cholinergic system has long been known to modulate other neurotransmission systems such as glutamatergic and GABAergic activity in the cerebral cortex (Pepeu and Blandina, 1998; Manns et al., 2001; McKay et al., 2007). Here, we showed that ATP/P2Y₁R could be another such modulating pathway in the brain as it could induce activity of cholinergic and glutamatergic genes. These conclusions, together

MOL #66506

with the other evidence presented here, suggest a functional significance for our findings,
now meriting further investigation.

MOL #66506

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MOL #66506

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MOL #66506

Footnotes

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Figure Legends

Fig 1. Mainly postsynaptic localization of P2Y₁Rs in cortical neurons. (A) Expression of P2Y₁R mRNA in rat brain cortex and in cortical neurons (CNs) cultured therefrom. Total RNA isolated from either source was analyzed, using RT-PCR with primers specific for the rat P2Y₁R and gel electrophoresis. Identification of each product was made by extraction from the gels and DNA sequencing. No products are seen from these RNA samples not subjected to RT, nor where template DNA was omitted (-ve control). Genomic DNA (5 ng per reaction) gives a positive control. (B) Cortical neuronal cultures, at 15 days in vitro (DIV), with or without Ara-C treatment to prevent proliferation of glia, were stained using antibodies to GFAP and to MAP-2 (a neuron-specific marker). After immunoblotting (*left panel*) or immunocytofluorescent staining (*right panel*), both results indicated that astrocytes were essentially absent from the Ara-C-treated cultures, thereafter routinely prepared thus. Bar: 40 μ M. Panels (C) - (E) were obtained in the presence of Ara-C to prevent glia proliferation. (C) Immunoblots showing the progressive expression of NF-200 (a neuronal differentiation marker), PSD-95 and P2Y₁R during the differentiation of the cultured cortical neurons. α -Tubulin served as an internal control for neuronal protein content. For each time-point all blots were run from the same sample. (D) Immunoblot, from cultures at 21 DIV, probed with anti-P2Y₁R (1 μ g/ml). Protein bands of ~60 kDa (the glycosylated P2Y₁R size) and ~42 kDa (the predicted P2Y₁R protein size) are detected, as in (C). The reaction of

MOL #66506

both forms is fully blocked by the peptide antigen (Ag, 5 µg/ml). (E) The cultures, at 21 DIV, were fixed and doubly stained using anti-P2Y₁R (green) plus *either* anti-PSD-95 (*upper panels*) or anti-SV48 (*lower panels*, presynaptic marker) antibody (both red), showing only dendrite regions. Punctate loci of P2Y₁R clearly co-localized with PSD-95 *or* with SV-48 are shown by arrowheads: the extent of this for each pair was analyzed across 20 fields (see text). Bar: 20 µm.

Fig 2. The P2Y₁R interacts via its C-terminal DTSL motif with the scaffold PSD-95. (A)

Immunoprecipitates (IP) from lysates of cultured cortical neurons (21 DIV) were obtained (see Materials and Methods) using antibodies to NR2A or PSD-95 or P2Y₁R. Blots derived therefrom were probed (IB) separately with anti-NR2A antibody (upper row), anti-PSD-95 antibody (middle row) or anti-P2Y₁R antibody (lower row). As controls, the corresponding normal serum replaced the IP antibody in the first lane (-ve) and total lysate (20 µg) alone was loaded in the last lane. In both directions in the grid, some P2Y₁R and NR2A are seen to be associated with PSD-95. (B) HEK 293T cells were co-transfected by PSD-95 cDNA plus P2Y₁R cDNA or plus cDNA of a C-terminally mutated construct (P2Y₁R_{DYSR} and P2Y₁R_{ΔC-term}) as illustrated. Each P2Y₁R carried an N-terminal HA epitope tag. Lysates (20 µg protein per lane) of each of these 3 cell types were tested in immunoblots using anti-HA

MOL #66506

antibody or anti-PSD-95 antibody, showing bands of the predicted sizes. Control blots with lysate from empty-vector-transfected cells did not react (-ve lane). (C) HEK 293T cells were co-transfected with PSD-95 and one of those 3 P2Y₁R constructs (all HA-tagged) as shown in the columns below the gel; IP and blotting were then performed on their lysates as in (A). IB was with anti-PSD-95 or anti-HA antibodies. The control (-ve lane) was as in (B) control. P2Y₁R is seen to be associated with PSD-95, but only when the DTSL motif is intact.

Fig 3. The C-terminal DTSL motif is required to link the P2Y₁R to PSD-95 at the plasma membrane. PSD-95 was tagged with GFP, while the P2Y₁R and its two mutated constructs (A, *top*) were N-terminally Myc-tagged, and each was expressed by transfection in HEK 293T cells. In (A), Myc-P2Y₁R and PSD-95-GFP were expressed separately and the former was reacted (without permeabilization) with Cy3-conjugated anti-Myc antibody. Confocal microscopy shows the wild-type P2Y₁R is at the cell membrane while PSD-95-GFP is largely intracellular. (B) When Myc-P2Y₁R was co-expressed with PSD-95-GFP, subsequent staining shows that the PSD-95-GFP has become co-localized with the receptor on the cell membrane (*lower panels*), with high coincident staining at the regions of high P2Y₁R content (*arrowheads*). This migration did not occur when PSD-95-GFP was co-expressed with Myc-P2Y₁R_{DYSR} (C, *lower*), nor with P2Y₁R_{ΔC-term}, (D, *lower*). In each

MOL #66506

case (B-D, *upper*) a control with the receptor co-transfected with the same vector expressing GFP alone shows that the GFP itself does not associate with P2Y₁R. Bar: 10 μ m.

Fig 4. Activation of P2Y₁Rs in cortical neurons induces Ca²⁺ mobilization and ERK phosphorylation. In all plots the values are normalized as multiples of the basal (no agonist) level. (A) A cortical neuron in a culture at 15 DIV, preloaded with Fluo-4, shows activation by the P2Y₁R agonist 2-MeSADP (100 μ M) with a transient rise (B) in cytosolic Ca²⁺ (internal green fluorescence). Bar: 10 μ m. (C) In a population of such neurons in 96-well plate format the potency of 2-MeSADP in Ca²⁺ activation was determined from readings (n = 4.5 x 10⁴ cells) as in (B). (D) The specificity of the Ca²⁺ mobilization induced by 2-MeSADP (10 μ M) was confirmed by pre-treatment with the P2Y₁R antagonists MRS2179 (100 μ M) or A3P5P (100 μ M), or with the general P2 antagonist suramin (100 μ M). (E) Immuno-blotting of extracts from the neuronal cultures after activation by three P2Y₁R agonists (each 100 μ M), or by TPA (100 nM, as a positive control). The transient phosphorylation of ERK-1 and -2 proteins is shown. Total ERK-1 and -2 proteins in each sample are shown as a loading control. *Lower panel*, quantitation from a set of such blots.

Fig 5. Temporal and spatial expressions of AChE in cortical neurons. (A) The AChE mRNA content and AChE enzymatic activity during differentiation were determined on

MOL #66506

extracts of cultured cortical neurons. The expression of the AChE protein was measured in parallel, from a set of its immunoblots (example shown; it gave 1 band, at ~68 kDa). Data are expressed as multiples of the basal activity (at 5 DIV). (B) Molecular forms of AChE in cortical neurons (at 25 DIV) were revealed by sucrose density gradient analysis of their extracts: the predominant form is G4 AChE (filled circles). This form of AChE was depleted specifically by reaction with anti-PRiMA antibody (squares). Data are expressed in arbitrary units. One of 4 representative plots is shown. (C) Neurons (21 DIV) were fixed and double-stained with anti-AChE antibody (red) and anti-PSD-95 antibody (green), seen in confocal microscopy. The lower panels show a higher magnification of the area marked with a rectangle: on dendrites there, AChE is partially co-localized with PSD-95 (*arrowheads*). Bar: 20 μ m.

Fig 6. Transcriptional regulation of AChE by the P2Y₁R. In all plots (except panel C) the values are normalized as multiples of the basal level (medium-treated control). (A) Cortical neurons (15 DIV) were treated with 50 μ M 2-MeSADP, 50 μ M ATP or 10 nM TPA for 12 h and the AChE mRNA expression was analyzed by real-time PCR. (B) Immunoblot showing the AChE protein content of such neurons, before or after treatment for 24 h with the P2Y₁R agonists shown (50 μ M), alone *or* (last 2 lanes) plus MRS2179 (250 μ M), a P2Y₁R-specific antagonist. GAPDH is the protein loading control. Quantitation from a set of 4 such blots

MOL #66506

(standardized for gel loading) is shown in the *right panel*. (C) A human AChE promoter sequence was tagged with the luciferase reporter gene (pAChE-Luc) and transfected into cultured cortical neurons (at 3 DIV), to provide measurements of the transcriptional activity during differentiation. The values are shown relative to the activity at 6 DIV. (D) Such pAChE-Luc-expressing neurons were exposed to ligands as in (B), followed by assay. (E) The pAChE-expressing neurons were exposed to the PKC activator TPA for 24 h, then assayed (*Left*). (*Right*), wild type Raf (Raf_{WT}) or its constitutively active mutant (Raf_{CAAX}) or the empty vector (pcDNA3) was co-transfected with pAChE-Luc, for similar assay. (F) The sites of the mutations (marked X) made at two Elk-1 sites in the AChE promoter, as described previously (Choi et al., 2003). (*Right*), each of these Luc-tagged constructs was transfected into cortical neurons, followed by exposure to 50 μ M ATP or 2-MeSADP for 24 h and a final transcriptional activity measurement. Differences marked are significant at $p < 0.05$ (*) or 0.01 (**).

Fig 7. Activation of P2Y₁Rs differentially induces synaptic gene expressions. (A) Immunoblots show the profiles of the expression in the cultured neurons of the glutamate receptor NR2A (~170 kDa) subunit, and of the cholinergic components ChAT (~65 kDa) and M1 mAChR (~50 kDa). Quantitations from a set of 4 such blots are shown in the *lower panel*, as multiples of the value at 5 DIV. (B) Neuronal cultures were treated with

MOL #66506

2-MeSADP (50 μ M, 24 h) alone, or plus MRS2179 (250 μ M). GAPDH is the protein loading control. Quantitation (n = 4 blots) is shown in the *lower panel*.

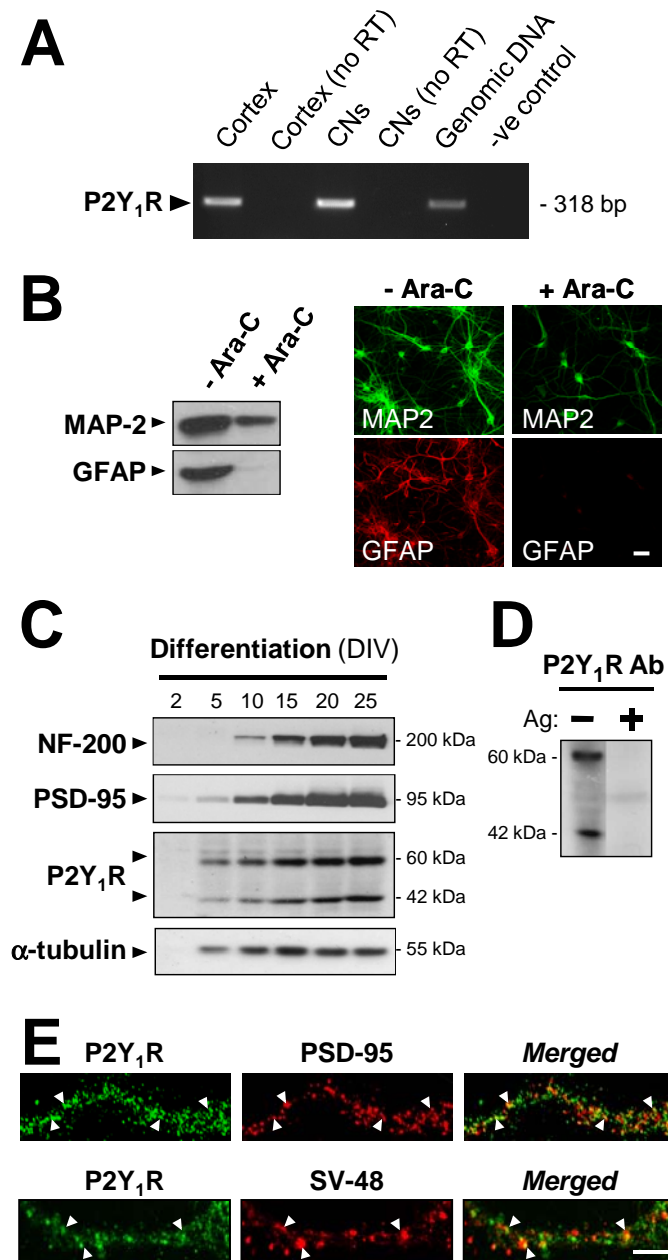


Figure 1

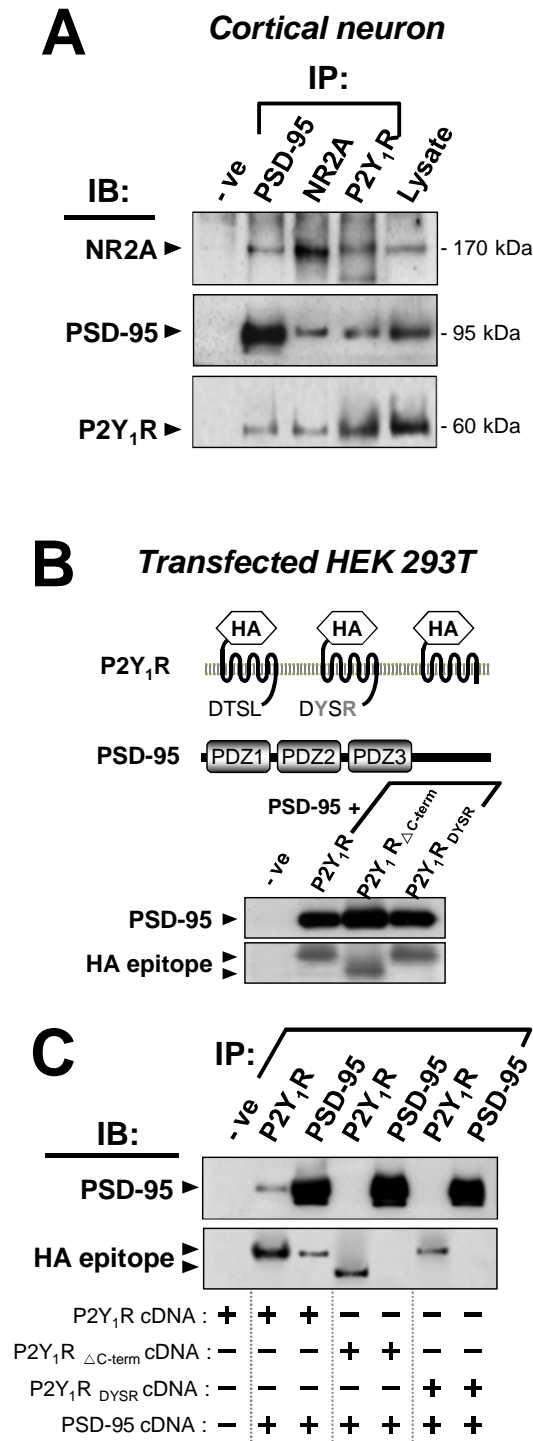


Figure 2

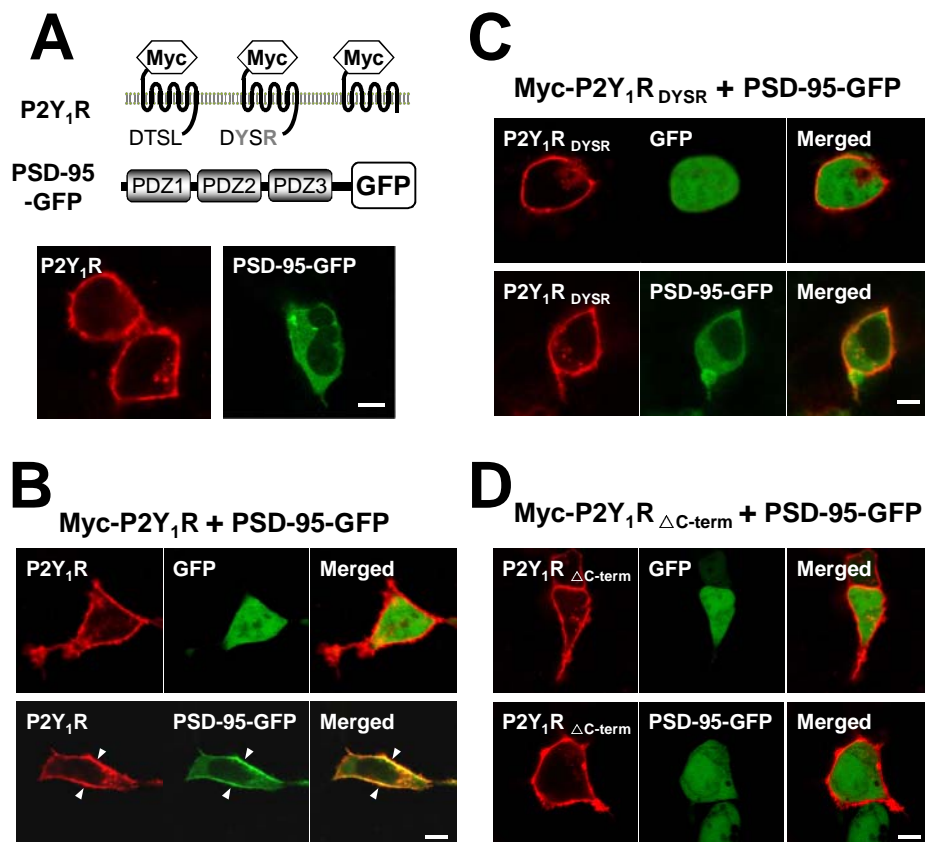


Figure 3

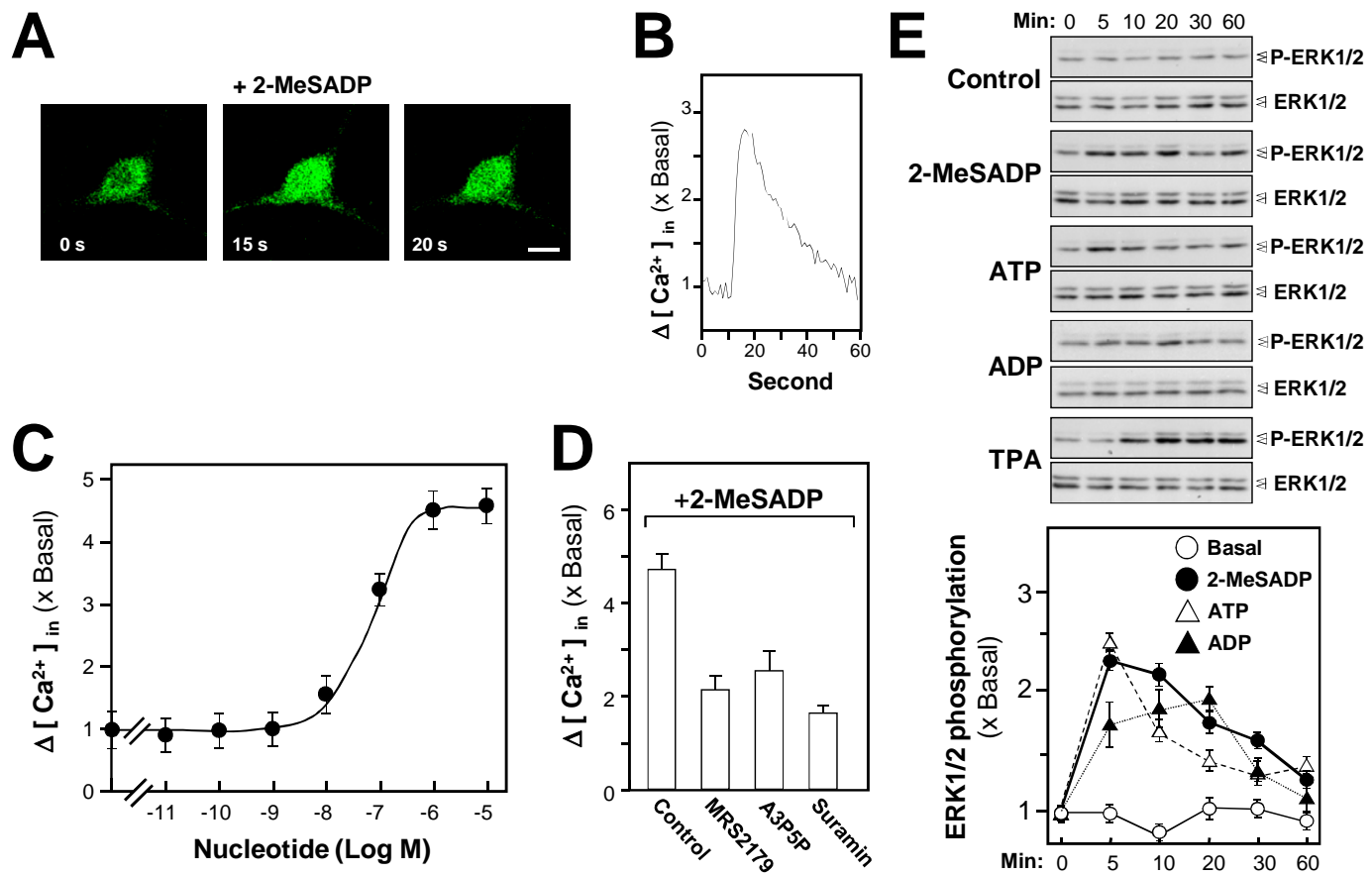


Figure 4

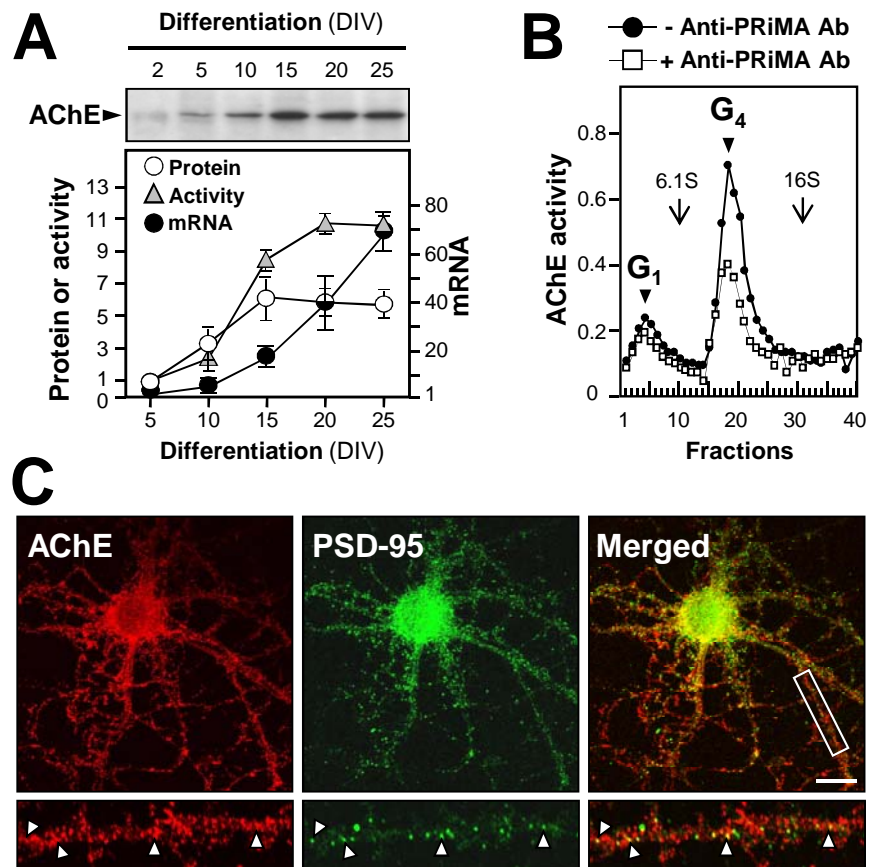


Figure 5

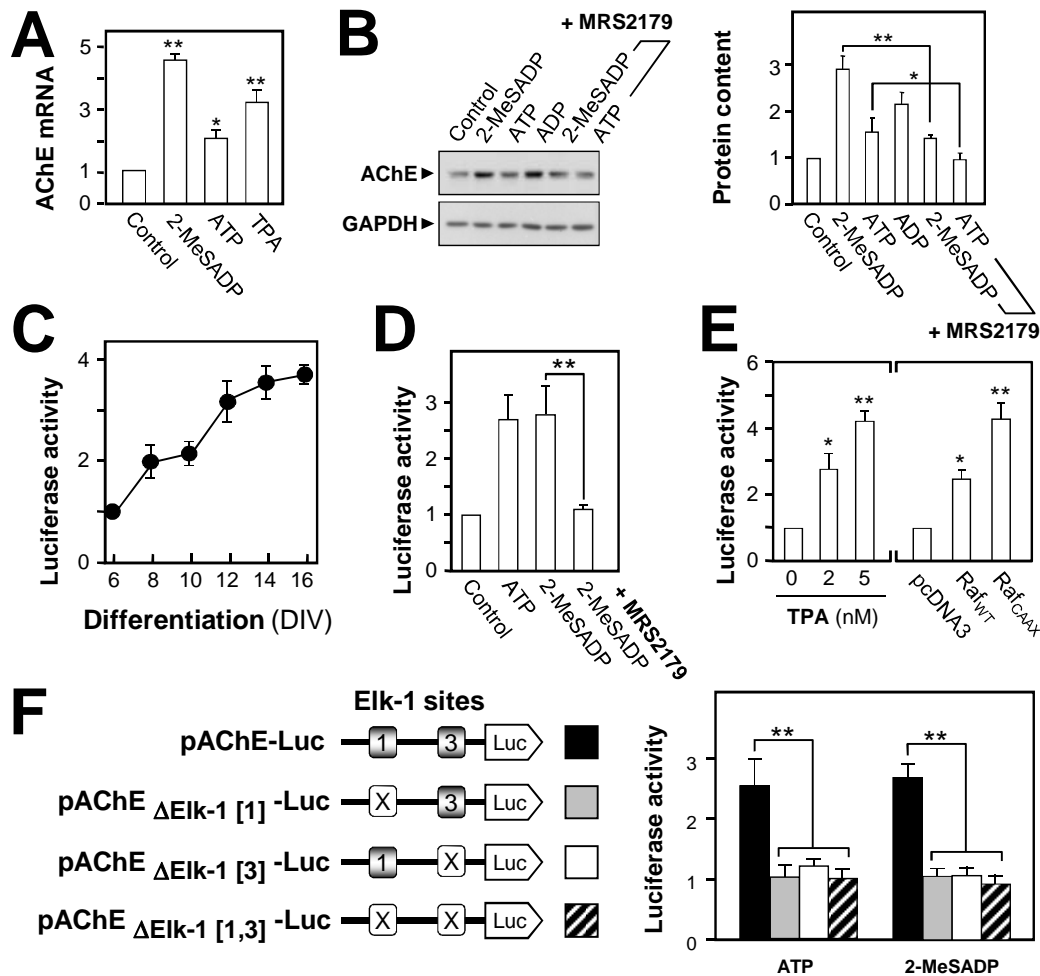


Figure 6

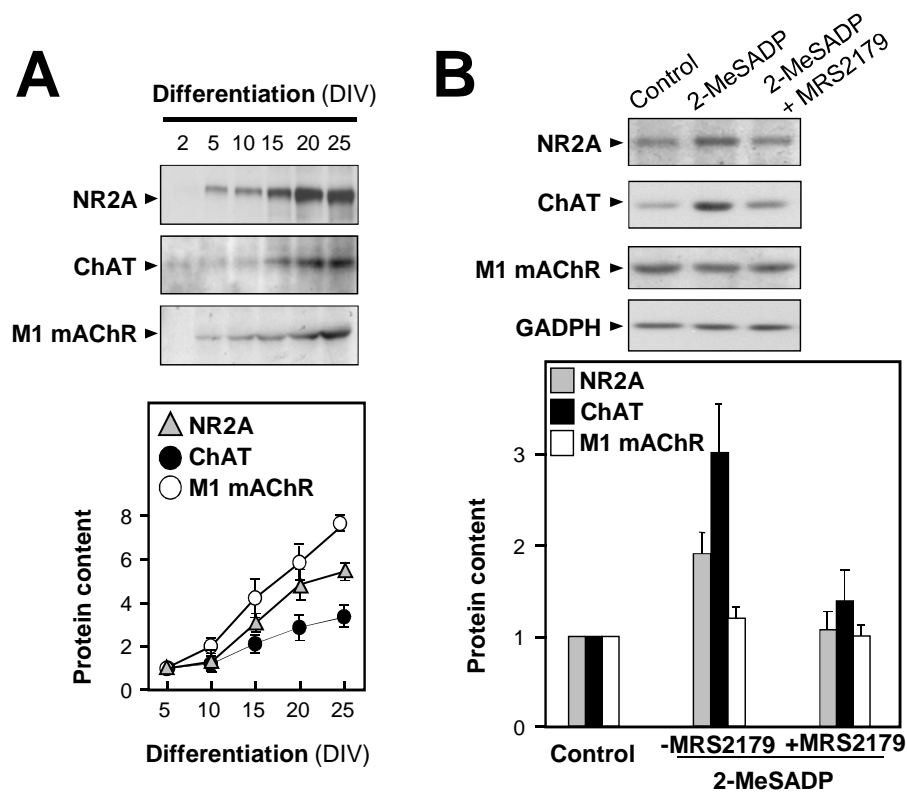


Figure 7