Activation of UTP-sensitive P2Y₂ receptor induces the expression of cholinergic genes in cultured cortical neurons: A signaling cascade triggered by Ca²⁺ mobilization and ERK phosphorylation


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Abstract

Adenosine 5'-triphosphate (ATP) functions as an extracellular signaling molecule that is co-stored and co-released with neurotransmitters at central and peripheral neuronal synapses. Stimulation by ATP up-regulates the expression of synaptic genes in muscle – including the genes for nicotine acetylcholine receptor (α-, δ- and ε-subunits) and acetylcholinesterase (AChE) – via the P2Y receptor (P2YR), but the trophic response of neurons to the activation of P2YRs is less well understood. We reported that cultured cortical neurons and the developing rat brain expressed different types of P2YRs, and among these the UTP-sensitive P2Y2R was the most abundant. P2Y2R was found to exist in membrane rafts and it co-localized with the post-synaptic protein PSD-95 in cortical neurons. Notably, agonist-dependent stimulation of P2Y2R elevated the neuronal expression of cholinergic genes encoding AChE, PRiMA (an anchor for the globular form AChE) and choline acetyltransferase, and this induction was mediated by a signaling cascade that involved Ca2+ mobilization and ERK1/2 activation. The importance of P2Y2R action was further shown by the receptor’s synergistic effect with P2Y1R in enhancing cholinergic gene expression via the robust stimulation of Ca2+ influx. Taken together our results revealed a developmental function of P2Y2R in promoting synaptic gene expression and demonstrate the influence of co-stimulation of P2Y1R and P2Y2R in neurons.
Introduction

Nucleotide receptors, or ATP receptors, which are involved in controlling different spatial and temporal functions in the body, are widely expressed in immune cells, the gastrointestinal tract, kidneys, the respiratory tract, bone and neurons (Abbracchio et al., 2006). There are two groups of ATP receptors: the ionotropic P2X receptors and the metabotropic P2Y receptors (P2YR). To date, eight subtypes of the P2Y receptor family have been cloned from mammals; these receptors exhibit differential sensitivity to adenine nucleotides ATP and ADP (P2Y$_{1,11,12,13}$Rs), uracil nucleotides UTP and UDP (P2Y$_{2,4,6}$Rs), and UDP-glucose (P2Y$_{14}$R). In the central nervous system (CNS), P2Y$_{1,2,4,6}$Rs have been detected in both neuronal and glial cells (Hussl and Boehm, 2006). The P2YRs are G protein-coupled receptors that activate phospholipase C (PLC) via $\alpha_{q/11}$, and stimulate or inhibit adenylyl cyclase via $\alpha_4$ and $\alpha_{i/o}$. These receptors can thus trigger distinct signaling cascades upon activation by ligands.

As a ligand ATP has been found to serve as a key neuro-modulatory factor that regulates neurological functions in both the CNS and the peripheral nervous system (PNS). Most of the studies on ATP signaling have focused on ATP/P2YRs. At the neuromuscular junction (NMJ), for example, ATP is co-released with acetylcholine (ACh) and acts as a neuro-modulator or neurotransmitter on post-synaptic muscle cells (Tsim and Barnard, 2002). ATP together with a neuropeptide, calcitonin gene-related peptide, can potentiate the post-synaptic ACh receptor (AChR) channel activity in the early phase of neuromuscular synaptogenesis in *Xenopus* (Fu WM, 1995).

Studies from our laboratory indicated that ATP, via P2Y$_1$R and P2Y$_3$R, plays a critical role in regulating gene transcription and in forming post-synaptic specializations at the NMJ (Choi et al.,
Moreover, the activation of P2Y<sub>1</sub>R triggered a mitogen-activated protein kinase signaling cascade, which subsequently up-regulated the expression of genes encoding acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) (Siow et al., 2010). In comparison, less is known about the extent to which other P2Y receptors are involved in regulating the expression of synaptic genes.

Uridine 5′-triphosphate (UTP) is another vital bio-molecule that elicits significant physiological responses in epithelial cell ion transport, hormone release and synaptic transmission (Lazarowski and Boucher, 2001). In mammalian cells UTP is a natural ligand for P2Y<sub>2</sub>R and P2Y<sub>4</sub>R (Burnstock G, 2007), which cannot be easily distinguished pharmacologically since both are activated by UTP. Previously, UTP and catecholamine were found in molar ratio of about 0.1:5 in chromaffin granules (Zimmermann H, 1994), and a ratio of 1:3-5 was observed for extracellular UTP/ATP both in resting and mechanically stimulated cell lines (Lazarowski et al., 2003). P2Y<sub>2</sub>R mediates neuronal differentiation via nerve growth factor/TrkA signaling (Arthur et al., 2005), performs neuro-protective, anti-apoptotic or pro-apoptotic functions, and modulates glutamate N-methyl-D-aspartate (NMDA) receptor currents or intracellular Ca<sup>2+</sup> (Kim et al., 2003; Cavaliere et al., 2005; Wirkner et al., 2007). Although many different lines of evidence have now demonstrated the physiological functions of UTP in diverse systems, the regulatory effects of the UTP-sensitive P2Y<sub>2</sub>R on neuronal gene expression remain largely unknown. Here, we present results of studies on the expression and cellular localization of P2Y<sub>2</sub>R in rat brain and cortical neurons and the receptor’s influence on the expression of synaptic genes.
Materials and Methods

**Materials**: Sprague-Dawley rats were provided by the Animal Care Facility at HKUST. Cell culture media were purchased from Invitrogen Technologies (Carlsbad, CA) and commercial antibodies were from Sigma (St. Louis, MO) except where specifically noted. Apyrase (Grade VII) and phorbol ester (TPA) were from Sigma. To ensure the purity of UTP and ATP, stock solutions (10 mM; Tocris Bioscience, UK) were pre-treated in buffer with 20 U/ml creatine phosphokinase and 10 mM creatine phosphate at room temperature for 90 min to remove all contaminating diphosphates, as described and validated previously (Simon et al., 2001; Choi et al., 2003). P2Y₁R-specific agonist MRS2365 ([(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo [3.1.0] hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt) and the P2Y₂R-specific agonists 2SUTP (2-ThioUTP tetrasodium salt), PSB1114 (4-Thiouridine-5′-O-(β,γ-difluoromethylene) triphosphate tetrasodium salt) and MRS2768 (Uridine-5′-tetraphosphate δ-phenyl ester) were purchased from Tocris Bioscience.

**Cell culture and drug treatment**: Primary cortical neurons were prepared as previously described (Siow et al., 2010). In brief, the cortex was dissected from brains of embryonic day 18 (E18; mixed sex) rats and incubated with 2.5% trypsin at 37°C for 15 min. Cultured neurons were grown in Neurobasal medium with B27 (Invitrogen) and 0.5 mM GlutaMAX and maintained in a humidified incubator with 5% CO₂ at 37°C. After 3 days in vitro (DIV), the glial-suppressive agent cytosine arabinoside (Ara-C, 2.5 μM; Sigma) was added. One-third of the medium was thereafter replaced by fresh culture medium every 4 days, and cultures were generally used after 15 DIV, except where
specified otherwise. Human embryonic kidney (HEK) 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum and maintained in a humidified incubator with 5% CO₂ at 37°C. In drug treatment assays, cultured cortical neurons at 15 DIV were first exposed for 1 hour to apyrase (1 U/ml) to remove endogenous nucleotides in the culture medium, and then washed once with Neurobasal medium. Drugs were next added in fresh culture medium for 15 to 24 hours and the treated cultures were collected for different analyses.

**mRNA analysis:** Total RNA was extracted from the cortex of embryonic and adult rats, or from cultured cortical neurons, using TRIZOL reagent (Invitrogen). RNA purity and quantity were determined by UV absorbance at 260 nm, and 5 μg of the RNA from cortex and neurons was reverse-transcribed by M-MLV reverse transcriptase (Invitrogen) in the presence of oligo-d(T) primers. Total cDNA from the cortex or cultured cortical neurons (500 ng each) was amplified by PCR using Taq DNA polymerase (1.25 U/reaction) in a reaction mixture containing these primers: P2Y₁R: 5'-CCT GCG AAG TTA TTT CAT CTA-3' (forward) and 5’-GTT GAG ACT TGC TAG ACC TCT-3’ (reverse); P2Y₂R: 5’-CTC TAC TTT GTC ACC ACC AGC GC-3’ (forward) and 5’-GTG GTC CCA TAA GCC GGT TTG-3’ (reverse); P2Y₄R: 5’-GGC ATT GTC AGA CAC CTT GTA-3’ (forward) and ‘5-AAG GCA CGA AGC AGA CAG CAA-3’ (reverse); and P2Y₆R: 5’-CGC TTC CTC TTC TTC TAT GCC AA-3’ (forward) and ‘5-GTA GGC TGT CTT GGT GAT GTG-3’ (reverse) (Moore et al., 2001; Fumagalli et al., 2003). In parallel we processed control samples using rat brain genomic DNA (5 ng/reaction) or RNAs (200 ng/reaction for cortex; 500 ng/reaction for cultured neurons) lacking reverse transcriptase. Amplifications were performed in a GeneAmp 9700.
PCR System (Applied Biosystems, Foster City, CA) for 40 cycles (94°C/45 sec; annealing 58°C/45 sec; 72°C/1 min). PCR products were resolved by electrophoresis on 1.5% agarose gels to confirm specific amplification.

*Real-time quantitative PCR:* Real-time PCR was performed using SYBR green master mix and Rox reference dye according to Roche’s instructions. The specific primers were: P2Y2R: 5’-CTC TAC TTT GTC ACC ACC AGC GC-3’ (forward) and 5’-GTG GTC CCA TAA GCC GGT TTG-3’ (reverse); AChE_T catalytic subunit: 5’-CTG GGG TGC GGA TCG GTG TAC CCC-3’ (forward) and 5’-TCA CAG GTC TGA GCC GGT TTC CTG-3’ (reverse) (Boudreau-Larivière et al., 2000); ChAT: 5’-GTG CAA AGA GCC ACC TGA G-3’ (forward) and 5’-TGT CAA CAA GGC TCG CTC C-3’ (reverse); proline-rich membrane anchor (PRiMA): 5’-TCT GAC TGT CCT GGT CAT CAT TTG CTA C-3’ (forward) and 5’-TCA CAC CAC CGC AGC GTT CAC-3’ (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5’-AAC GGA TTT GGC CGT ATT GG-3’ (forward) and 5’-CTT CCC GTT CAG CTC TGG G-3’ (reverse) (Lee et al., 2004). SYBR green signal was detected on the Mx3000ptm multiplex quantitative PCR platform (Stratagene, La Jolla, CA). Transcript expression levels were measured using the ΔΔCt value method (Winer et al., 1999), where values were normalized by the internal control GAPDH in the same sample. PCR products were examined as above and using melting curve analysis to confirm specific amplification.

cDNA plasmids and transfection: Rat P2Y2R cDNA was sub-cloned into pcDNA4 His-Max expression vector using Pfx (Invitrogen) to form N-terminal His-Xpress-P2Y2R (HX-P2Y2R).
HEK293T cells were grown in culture dishes for 24 hours before transfecting with plasmids using calcium phosphate precipitation (Choi et al., 2003); the transfection efficiency of HEK293T cells was usually ~90%. The Xpress tag contained the amino acid sequence of DLYDDDDK.

**Immunoblotting and phosphorylation studies:** Cultured cortical neurons were collected in lysis buffer 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. For phosphorylation analysis (Siow et al., 2010), cultures were serum-starved for 3 hours before applying reagents. Supernatants of the final lysates prepared from these samples were loaded at 10 μl per gel-lane in differentiation and phosphorylation experiments, while 10 μg of protein was loaded per lane in agonist-treatment assays. Proteins were separated on 8%-12% SDS-polyacrylamide gels and electroblotted onto nitrocellulose filters for 16 hours. After blocking with 5% non-fat dried milk in TBST (20 mM Tris.HCl, 137 mM NaCl, 0.1% Tween-20, pH 7.6), membranes were exposed to various primary antibodies (at noted concentrations): anti-P2Y2R (1:1000; APR-010; Alomone Labs, Israel), anti-AChET (1:500; Clone E19; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-ChAT (1:2000; Millipore, Billerica, MA), anti-PSD-95 (1:5000; Millipore), anti-neurofilament 200 (NF-200; 1:2000, Sigma), anti-flotillin-2 (1:1000; BD Biosciences, Franklin Lakes, NJ), anti-PRiMA (1:1000, self-generated; 24), anti-mAChR M1 (1:1000; Sigma), anti-phospho-ERK and anti-ERK (1:5000; Cell Signaling Technology, Danvers, MA), and anti-GAPDH and anti-α-tubulin (1:10,000; Abcam, Cambridge, UK). Proteins bound by primary antibodies were detected using corresponding peroxidase-conjugated secondary antibodies (1:5000, Invitrogen) and enhanced
chemiluminescence (ECL protocol, GE Healthcare, Piscataway, NJ). The labeling intensities of protein bands from control and agonist-stimulated samples, run in the same gel, were compared by densitometry within the range of a calibrated density/response curve. GAPDH or α-tubulin were included as visual loading controls or used to correct the densities before plotting.

**Immuno-fluorescent staining:** Cultured cortical neurons or HEK293T cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. After washing with 50 mM ammonium chloride (NH₄Cl) for 25 min, cultures were permeabilized by 0.1% Triton X-100 in PBS for 10 min and then blocked with 5% BSA in PBS for 1 hour at room temperature. The following primary antibodies were next applied for 16 hours at 4°C: anti-P2Y₂R (1:200; APR-010, Alomone Labs), anti-Xpress (1:1000; Invitrogen), Cy3-conjugated anti-GFAP (1:2000; Sigma), anti-MAP-2 (1:500; Sigma), and anti-PSD-95 (1:500; Millipore). After PBS washes (3 x 15 min), cultures were labeled with Alexa 488- or 555-conjugated secondary antibodies (Invitrogen) plus the nuclear stain TO-PRO-3 (1:500, Invitrogen) for 3 hours at room temperature. After washing thrice more with PBS (15 min each), samples were dehydrated serially in ethanol and mounted with fluorescence mounting medium (Dako, Carpinteria, CA). Confocal fluorescence microscopy (Zeiss LSM 510) was used with excitation (Ex) 488 nm/emission (Em) 500-535 nm for green color, Ex 543/Em 560-615 nm for red color, and Ex 640/Em 660-750 nm for pseudo-color (TO-PRO-3).

**Membrane raft preparation:** Membrane rafts were prepared as previously described (Xie et al.,
In brief, adult male rat brains or cortical neurons (20 DIV) were homogenized (1 g/10 ml) in buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM benzamidine HCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 10 sec six times at 9,500 rpm (IKA, Staufen, Germany) and then resuspended in buffer A. The suspensions were sonicated at low intensity three times for 0.5 sec with 30-sec intervals to avoid heating and the samples were centrifuged at 500 xg for 5 min to remove cell debris and nuclei. The post-nuclear extracts of tissues or cells were centrifuged at 180,000 xg for 30 min in a Sorvall TST 60.4 rotor and the resulting pellet was washed and resuspended by sonication in 600 μl of buffer A containing 5% glycerol. This preparation was used as the total membrane fraction. For analysis of membrane rafts, a 500 μl sample was incubated with 0.5% Triton X-100 on ice for 1 hour and applied to a discontinuous flotation gradient. The sample was mixed with an equal volume of 80% sucrose in buffer A, placed at the bottom of a 4 ml ultracentrifugation tube, and overlaid with 2.4 ml of buffer A containing 30% sucrose and with 0.6 ml of buffer A containing 5% sucrose. The resulting discontinuous gradient was spun at 50,000 rpm in a Sorvall TST 60.4 rotor for 18 hours at 4°C and then twenty fractions (190 μl each) were collected from the top and used for Western blotting.

Fluorometric measurement of \(Ca^{2+}\) mobilization: Cultured HEK293T cells transfected with pcDNA3 (mock) or P2YR cDNAs were seeded into black-walled, clear-bottomed 96-well culture plates. Cells were labeled with 2 mM Fluo 4-AM (Invitrogen) at 37 °C for 1 hour and then the medium was replaced by 150 μl Hank's balanced salt solution (HBSS). Cortical neurons (15 DIV) were also labeled by the same procedure, except that tetrodotoxin (100 nM; Sigma) was added to
the buffer to obtain a stable baseline reading. All agonists were prepared in HBSS, and 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 fluorescence intensity relative to the basal (untreated) value and analyzed with GraphPad Prism 3.

**Data analysis:** Gel documentation and relative quantification were performed with the IS1000 Digital Imaging System. To obtain co-localization ratios of proteins in cultured neurons, twenty neurons that appeared morphologically healthy in phase-contrast were randomly selected from each double-immunolabeled coverslip; all immuno-positive puncta present in 50 μm dendrite segments were counted, with only puncta larger than 10 pixels being used in order to avoid including the background staining. The co-localization ratio (%) for each pair of immuno-positive puncta was calculated thus: [number of puncta double-immunostained/number of total puncta immunostained with one of the two antibodies] x 100. On average 80 puncta were analyzed per neuron and statistical analyses were by two-tailed, unpaired, one-way ANOVA. Data are plotted as mean ± 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.

**Other assays:** Protein concentration, AChE enzymatic activity and separated AChE molecular forms on sucrose density gradients were performed as previously described (Chen et al., 2011).
Results

**P2Y₂R in brain and cultured cortical neurons**

To address the functional role(s) of P2Y₂R in the brain, we began by investigating the existence of various P2YR mRNAs in E18 and adult rat cerebral cortices by RT-PCR analysis (Fig. 1A). P2Y₁R, P2Y₃R, P2Y₄R and P2Y₆R were detected in both E18 and adult cortex, consistent with previous reports (Webb et al., 1998; Moore et al., 2001; Siow et al., 2010). Quantitative real-time PCR further revealed that the relative levels of these different P2YR mRNAs increased from embryonic to adult stages (Fig. 1B), with the P2Y₂R mRNA showing the maximal change during development; expression of the P2Y₂R transcript in adult was ~5-fold higher than at the E18 stage. Next, we examined the expression of P2Y₂R protein with a commonly-used commercial anti-P2Y₂R antibody, whose target epitope is located in the third intracellular loop of P2Y₂R. To test for the antibody’s specificity, P2Y₂R was tagged with Xpress epitope and transfected into HEK293T cells for double immuno-labeling with anti-P2Y₂R and anti-Xpress antibodies. The overlap in fluorescent staining by these two antibodies indicated that anti-P2Y₂R recognized the expressed P2Y₂R in the transfected cells (Fig. 1C), and pre-incubation of the probing solution with the antigenic peptide from P2Y₂R blocked the fluorescent signal from anti-P2Y₂R but not anti-Xpress antibody (Fig. 1C). In Western blotting, P2Y₂R protein was detected as an ~65 kDa band in extracts of P2Y₂R-transfected HEK293T cells, and this staining was also abolished when anti-P2Y₂R antibody was pre-incubated with blocking peptide (Fig. 1D). These results indicated that the anti-P2Y₂R antibody specifically recognized its target. Anti-P2Y₂R also stained P2Y₂R in rat brain lysates, where the protein’s expression increased from embryonic to adult stages (Fig. 1E); here α-tubulin
and GAPDH served as loading controls. The receptor size of ~65 kDa in brain lysates was in accord with that in heterologous cells above and from a previous report (Roberts et al., 2006). These results indicated that P2Y2R was present in cortical neurons and up-regulated during neuronal differentiation.

Although P2Y2R levels increased during brain development (above), this up-regulation could have occurred in both glia and neurons. To specifically examine neuronal P2Y2R, primary cortical neurons were used as a model (Brewer GJ, 1995; Siow et al., 2010). In preparations of neuron cultures, glial cells can often be found, and astrocytes are known to express P2YR subtypes such as P2Y3R and P2Y4R (Abbracchio and Ceruti, 2006; Fischer and Krügel, 2007). Thus, to obtain highly pure cortical neuron cultures, Ara-C (a DNA synthesis inhibitor) was added on 3 DIV to help eliminate the contamination by glial cells. With Ara-C-treatment no fluorescent signal was detected for GFAP (a glial marker) in mature cortical neuron cultures, while the signal for MAP-2 (a neuronal marker) was unaffected (Fig. 2A). These results indicated that our cortical neuron preparations were suitable for further neuron-selective studies. First, standard RT-PCR showed that P2Y2R mRNA was robustly expressed in mature cortical neurons (Fig. 2B). Here again rat brain genomic DNA served as a positive control and RNA without reverse transcription (-RT) served as a negative control. Staining with anti-P2Y3R next revealed the expression profile of P2Y3R during the differentiation of cortical neurons (5 DIV to 25 DIV): the P2Y3R protein level increased throughout this period (Fig. 2C upper panel). The proper differentiation of these neuronal cultures was confirmed by the parallel up-regulation of two markers, NF-200 (200 kDa; a neuronal
cytoskeleton protein) and PSD-95 (95 kDa; a neuronal scaffolding protein). Densitometric analysis of protein bands and normalization against the internal control GAPDH demonstrated that the levels of PSD-95 and NF-200 were elevated ~13-fold and ~11-fold, respectively, and that the expression of P2Y2R went up ~7-fold during the differentiation process (Fig. 2C lower panel).

Cellular localization of P2Y2R

To investigate the cellular distribution of P2Y2R, cortical neurons were labeled with the anti-P2Y2R antibody. The resulting immuno-fluorescent staining revealed a synaptic localization of P2Y2R (Fig. 3): in mature cortical neurons, P2Y2Rs (green) were found on the cell body but were also enriched in the dendrites, where they partially co-localized with the post-synaptic marker PSD-95 (red). Quantification of this co-localization showed that 47.5% ± 2.5% of P2Y2R puncta overlapped with PSD-95 puncta (with respect to total PSD-95 puncta).

Over the past decade several lines of evidence have suggested that special cholesterol- and sphingolipid-rich microdomains in the plasma membrane – termed lipid rafts – play diverse roles in cellular signaling (Suzuki T, 2002; Tsui-Pierchala et al., 2002), and that cytoskeletal and scaffold proteins are often anchored to these rafts (Brückner et al., 1999). Because synaptic roles have also been uncovered for raft proteins, it was of interest to test whether P2Y1R and/or P2Y2R was associated with membrane rafts. This restriction localization of the two receptors in this micro-domain would provide spatial advantage in mediating the synergistic action that has been revealed in the rest of study. Extracts of adult cortex and cortical neurons (30 DIV) were prepared.
and separated by a discontinuous sucrose density gradient to isolate membrane rafts. P2Y₁R (~60 kDa) and P2Y₂R (~65 kDa) were present in the lipid raft-enriched fractions (fractions 5-7), identified by the raft-marker flotillin-2 (~47 kDa), in both adult rat cortex (Fig. 4A left panel) and in mature cortical neurons (Fig. 4A, right panel); in these immuno-blots total lysates were used as positive controls. In addition to P2Y₁R and P2Y₂R, our raft fractions contained other well-known synaptic markers, including AChE (~68 kDa), mAChR M1 (~50 kDa), NMDA receptor subunits NR1 (~130 kDa) and NR2A (~170 kDa) (Besshoh et al., 2005), and the post-synaptic protein PSD-95 (~95 kDa) (Wong and Schlichter, 2004). Densitometry indicated that ~45% of P2Y₁R in adult cerebral cortex was associated with membrane rafts, while ~86% of P2Y₁R in cultured cortical neurons was in rafts (Fig. 4B). For P2Y₂R, similar findings were observed that ~38% of receptor was associated with membrane rafts in adult cerebral cortex and ~83% of receptor in cultured cortical neurons was in rafts (Fig. 4B). These results demonstrated, for the first time, the cellular and subcellular localization of P2Y₁R and P2Y₂R in neurons and adult brain and suggested a potential role of the UTP-responsive P2Y₂R receptors, like P2Y₁R, in regulating neuronal function(s).

Regulation of cholinergic gene expression by P2Y₂R

P2YRs are known to regulate the expression of the cholinergic genes in muscle (see above). Thus, here we chose to analyze the expression of AChE, the AChE-anchor PRiMA, and ChAT, and, using a pharmacological approach, tested different P2Y₂R-selective agonists. First, HEK293T cells were transfected with P2Y₂R cDNAs to examine agonist specificity in the Ca²⁺ mobilization response. In

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control cells transfected with pcDNA3 (mock), addition of ATP and UTP slightly induced Ca\textsuperscript{2+} elevation, suggesting that low levels of endogenous P2Y receptors were present in HEK293T cells (Fig. 5A). Following the overexpression of P2Y\textsubscript{2R}, the Ca\textsuperscript{2+} signals elicited by ATP and UTP were both higher than that seen in the mock-transfected controls (as expected), and with the addition of P2Y\textsubscript{2R}-specific agonists (2SUTP, PSB1114 and MRS2768) these responses were even higher (Fig. 5A). In other controls, the P2Y\textsubscript{1R}-specific agonist MRS2365 did not trigger Ca\textsuperscript{2+} elevation in mock- or P2Y\textsubscript{2R}-transfected cultures, and the P2Y\textsubscript{2R}-specific agonists failed to elicit a response in P2Y\textsubscript{1R}-transfected cultures (Fig. 5A). The dose-response curves of these agonists (MRS2365 for activating P2Y\textsubscript{1R}, 2SUTP, PSB1114 and MRS3768 for activating P2Y\textsubscript{2R}) were showed in Supplementary Figure 1. Based on these pharmacological studies, the P2Y\textsubscript{2R}-specific agonists 2SUTP, PSB1114 and MRS2768 were employed for the specific activation of P2Y\textsubscript{2R} in cortical neurons.

Treatment of cortical neurons with ATP or UTP enhanced the mRNA expression of the cholinergic genes AChE, ChAT and PRiMA to different extents (Fig. 5B). Exposure to P2Y\textsubscript{2R}-specific agonists 2SUTP, PSB1114 and MRS2768 also significantly elevated the mRNA levels of these cholinergic genes, with the increases quantified to be as much as 3-4-fold (Fig. 5B). In these assays TPA served as a positive control. In parallel we also determined the changes in protein expression by Western blotting. Upon treatment with UTP or P2Y\textsubscript{2R}-specific agonists, the protein levels of AChE, ChAT and PRiMA were up-regulated to different extents (Fig. 5C). ATP and TPA were used as positive controls, and GAPDH was immuno-blotted as a normalization control for protein quantification.
Because P2Y2R activation increased the AChE protein expression, it was of interesting to test if the enzymatic activity and molecular form(s) of AChE were also altered. However, examination of lysates from drug-treated cortical neurons indicated that the stimulation of P2Y2R failed to influence either the enzymatic activity or the molecular forms of AChE (Supplementary Figure 2), which is consistent with our previous studies (Siow et al., 2010). The above results suggested that the activation of P2Y2R enhanced the expression of different cholinergic genes in cortical neurons.

P2YRs belong to a distinct subset of G-protein-coupled receptors, which five of them acts through Gq-coupled signaling cascade. Activation of P2Y2R can stimulate PLC and increase Ca2+ from intracellular stores (Van Kolen and Slegers, 2006), and the UTP-sensitive P2Y2R can trigger downstream MAP kinase-dependent signaling in neuronal PC12 cells and endothelial cells (Bowden et al., 1995). Such a UTP-mediated cell signaling cascade has also been reported at the NMJ (Tung et al., 2004). Therefore, we examined whether this ‘classical’ signaling was initiated by P2Y2R in cortical neurons. Cortical neurons were pre-loaded with the Ca2+ indicator Fluo 4-AM, and upon the addition of UTP or PSB1114 the intracellular Ca2+ was found to be sharply elevated relative to controls (Fig. 6A). For this response the Ca2+ ionophore A23187 served as a positive control.

The activation of P2Y3R in cultured cortical neurons also led to the phosphorylation of extracellular regulated kinases 1 and 2 (ERK1, ~44 kDa; ERK2 ~42 kDa), which was shown by Western blotting (Fig. 6B): treatment with UTP or PSB1114 induced the phosphorylation of ERK1/2, with the peak response occurring at 5 min and then gradually declining until 60 min. TPA served as a positive control.
control for the stimulation of ERK1/2 phosphorylation. The above results suggested that the activation of P2Y2R can trigger Ca^{2+} mobilization and induce ERK1/2 phosphorylation in cultured cortical neurons, which is consistent with previous findings with P2Y2R in muscle and other cell types.

Lastly, to ask if Ca^{2+} and MAP kinase-dependent pathways participate downstream from P2Y2R in regulating the expression of synaptic genes in cortical neurons, we used the pharmacological inhibitors BAPTA-AM (Ca^{2+} chelator), Gö6976 (PKC inhibitor) and PD98059 (MEK1 inhibitor). To confirm the effectiveness of these blockers, cultured cortical neurons were treated with the inhibitors for 1 hour before activating P2Y2R by the receptor-specific agonist PSB1114. Our results showed that all three inhibitors significantly reduced PSB1114-induced ERK phosphorylation (Fig. 6C). With regards to gene regulation, this drug pre-treatment did not affect the basal expression of AChE, ChAT or PRiMA mRNAs (Fig. 6D), but the inhibitors abolished the up-regulation of cholinergic genes by UTP and PSB1114 (Fig. 6D). These data suggested that the regulatory effects of P2Y2R on the expression of three different cholinergic genes in cortical neurons were mediated by a Ca^{2+} and MAP kinase-dependent signaling pathway.

**Synergistic action of P2Y1R and P2Y2R**

Our current results suggested a functional role of P2Y2R in cortical neurons by demonstrating its involvement in regulating cholinergic gene expression. Previously we found that cortical neurons also contain P2Y1R that can stimulate the expression of AChE and ChAT (Siow et al., 2010). This
raised an interesting question about the significance of two P2Y receptors co-existing in cortical neurons. The co-existence of P2Y$_1$R and P2Y$_2$R in the same post-synaptic terminal were partially supported by our previous and current findings of double immuno-staining of PSD-95 with P2Y$_1$R (Siow et al., 2010), and PSD-95 with P2Y$_2$R (Fig. 3). Given their distinct ligand selectivity (ATP/ADP for P2Y$_1$R and UTP for P2Y$_2$R) as well as their synaptic localization in neurons, we hypothesized a synergistic action between P2Y$_1$R and P2Y$_2$R. To test this, cortical neurons were co-treated with the P2Y$_1$R-specific agonist MRS2365 and the P2Y$_2$R-specific agonists PSB1114 and MRS2768 and then analyzed for Ca$^{2+}$ mobilization and gene expression. A synergy in P2Y$_1$R and P2Y$_2$R actions was first demonstrated by a robust increase of intracellular Ca$^{2+}$ (Fig. 7A): the activation of P2Y$_1$R alone (by MRS2365) or P2Y$_2$R alone (by PSD1114) caused an ~2-fold increase in Ca$^{2+}$ level relative to control, while the co-application of the two receptor agonists produced an ~5-fold elevation of Ca$^{2+}$. Next, at the gene expression level, the mRNAs encoding AChE, ChAT and PRiMA increased ~4-fold after P2Y$_1$R activation, which was similar to that seen with the stimulation of P2Y$_2$R (Fig. 7B). However, treatment with MRS2365 + PSB1114 (or MRS2365 + MRS2768) induced gene expression ~8-fold (Fig. 7B). The co-activation of P2Y$_1$R and P2Y$_2$R (MRS2365 + PSB1114 or MRS2365 + MRS2768) also led to higher expression of the proteins encoded by the cholinergic genes, ~7-fold more than that achieved with the stimulation using MRS2365 or PSB1114 alone (Fig. 7C). These results suggest that the co-activation of two P2Y receptors under physiological conditions (with ATP or ADP for P2Y$_1$R, and UTP for P2Y$_2$R) can produce synergistic responses in terms of receptor signaling and gene expression in cortical neurons, which might account for the functional necessity of two P2Y receptors being localized at the
post-synaptic region in these neurons.

Discussion

Association of P2Y2R with synaptic regions and membrane rafts

P2Y2R is expressed in various mammals (Abbracchio et al., 2006) and presents diverse tissue-specific roles in epithelial cells, cardiomyocytes, osteoblasts, and cells of the PNS and CNS (Kunapuli and Daniel, 1998; Dubyak GR, 2003; Jørgensen NR, 2005; Weisman et al., 2005). In the CNS, P2Y2R has been found to localize on neurons in hippocampus, prefrontal cortex, hypothalamus and spinal cord (Abbracchio et al., 2006). We observed a developmental increase in P2Y2R levels, which might suggest specific functions during brain development (Cheung et al., 2003; Franke and Illes, 2006). Our cultures of rat cortical neurons also showed an elevation in the amount of P2Y2R protein during differentiation, an increment that could be caused by an increase in the number of synapses that accompanies differentiation, as previously reported (Muramoto et al., 1993; Zhu and Kimelberg, 2001).

By immuno-fluorescent staining P2Y2R was localized at post-synaptic regions. Post-synaptic P2YRs are thought to be responsible for initiating signal transmission, whereas the pre-synaptic receptors are primarily involved in modulating neurotransmitter release (Cunha and Ribeiro, 2000). We further found that both P2Y1R and P2Y2R was concentrated in membrane lipid-rafts, as were several other post-synaptic proteins, including mAChR M1, NR1, NR2A and PSD-95. The specific raft-association of the synaptic proteins is consistent with previous findings (Hering et al., 2003).
Indeed, recent reports provide evidence that membrane rafts contribute to the maintenance of synapses, receptor stability and synaptic transmission (Tsui-Pierchala et al., 2002; Hering et al., 2003). Thus, raft-associated P2Y2R may imply a post-synaptic signaling function in neurons, which might participate in the co-regulation of AChE and NR2A by P2Y1R (Siow et al., 2010). More importantly, the co-existence of P2Y1R and P2Y2R in membrane lipid-rafts would provide a spatial advantage in governing the synergistic action of two receptors in mediating the signaling transduction and gene expression.

The sources of ATP and UTP in the brain

Under physiological conditions, ATP is released in response to myriad stimuli (Novak I, 2003). ATP is a co-transmitter in synaptic vesicles together with neurotransmitters, and also be released by neuronal neighbors such as astrocytes (Lazarowski and Harden, 1999). The extracellular ATP concentration in culture medium varies with different types of cells: from 1 nM in primary astrocyte cultures to 3.1 nM in PC12 neuronal cell cultures. For UTP the source of release could be many kinds of cells, including epithelial cells, nerve cells and astrocytes (Lazarowski and Harden, 1999). The extracellular UTP concentrations in culture medium of different cell types are also in nM range, e.g., 2.8 nM in human bronchial epithelial cell, <1 nM in primary astrocytes, and 4.6 nM in PC12 neuronal cells. Recently, a 1:3-5 ratio was reported for extracellular UTP/ATP under resting and mechanically-stimulated conditions in many cell lines (Lazarowski et al., 2003). These elements may comprise the core components of an ATP signaling system operative in neurons. It is possible that the actual ambient concentrations of ATP and UTP measured in cultures are also
underestimated because of the presence of ecto-nucleotidases which degrade extracellular ATP and UTP to ADP and UDP, respectively. Nevertheless, whether UTP is released from neurons or glia, the presence of P2Y$_2$R in cortical neurons indicates the importance of signaling by this receptor of UTP in neurons. One of an interesting issue is that ATP is the agonist for both P2Y$_1$R and P2Y$_2$R, which may theoretically produce the simultaneous receptor activation in producing synergistic effect. However, in our results, the stimulatory effects of ATP, UTP and other receptor-specific agonists in Fig. 5B and 5C were rather similar. This might imply that the co-activation of two receptors by ATP could not provide the maximum efficacy of receptor activation for synergistic effect, as the drug potency of P2Y$_1$R is MRS2365 > 2-MeSADP > ADP > ATP, while that of P2Y$_2$R is PSB1114 > MRS2768 > UTP ~ ATP.

**MAP kinase-dependent signaling pathway induced by P2Y$_2$R activation**

The signaling pathway of P2Y$_2$R is well-documented (Burnstock G, 2007). P2Y$_2$R couples to PLC-β via Gα$q$ to mediate IP$_3$ production, which results in the release of intracellular Ca$^{2+}$ and activation of PKC. Treatment of UTP (P2Y$_2$R and P2Y$_4$R agonist) and PSB1114 (P2Y$_2$R-specific agonist) boosted intracellular Ca$^{2+}$ mobilization. To further elaborate the UTP-induced signalling, we investigated ERK phosphorylation. MAP kinases are a group of serine-threonine kinases that transduce the external signals into intracellular biological events. The MAP kinase-dependent pathway involving Raf/MEK/ERK responds to the activation of tyrosine kinase receptors and also to P2Y$_1$R- or P2Y$_2$R-signaling in muscle cells and in P2Y$_1$R-mediated signaling in neurons (Choi et al., 2001; Tung et al., 2004; Siow et al., 2010). In line with the previous reports, UTP and PSB1114
(P2Y2R-specific agonists) induced robust phosphorylation of ERK1 and ERK2 in a time-dependent manner. Our results suggest that MAP kinase signaling is activated by Ca2+ and PKC and it appears that nucleotide-mediated P2YR activation initiates similar signaling cascades with similar cellular consequences in muscle and neurons (Tu et al., 2000; Choi et al., 2003; Milosevic et al., 2006; Siow et al., 2010). In our results, the responses of UTP in Ca2+ mobilization and Erk phosphorylation in Fig. 6 were higher than that of P2Y2R-specific agonist PSB1114. This might be attributed to the simultaneously activation of P2Y4R by UTP in the cultures. From our preliminary results, P2Y4R was also existed in mature cortical neurons. Since activation of P2Y4R could also trigger IP3 formation and Ca2+ mobilization via Gq/11 coupling (Unpublished data). This is a critical reason for using the P2Y2R-specific agonists for testing rather than UTP in the current study.

Activation of P2Y2R in cultured cortical neurons elevated the expression of several synaptic mRNAs and proteins, including those encoded by cholinergic ACHE, CHAT and PRIMA genes. According to our previous study, a family of transcription factors called c-Ets participated in mediating the gene transcription effect of P2Y1R via Raf/MEK/ERK pathway in muscles (Choi et al., 2003) and in cortical neurons (Siow et al., 2010). For P2Y2R, the similar regulatory pathway was also reported in muscles (Tung et al., 2004) and in cortical neurons (Supplementary Figure 3). By using an AChE promoter tagged with a luciferase reporter, the treatment of UTP and different P2Y2R agonists (2SUTP, PSB1114 and MRS2768) could stimulate the transcriptional activity of AChE promoter in transfected cortical neurons. On the other hand, the deletion of binding sites of c-Ets transcription factor (Elk-1) partially abolished the inductive effects of those agonists.
(Supplementary Figure 2). Therefore, this signaling cascade (mediated by c-Ets transcription factor in regulating AChE expression) might also be applied to PRiMA and ChAT. To address this issue, the promoter region of PRiMA and ChAT genes (~3kb upstream of the first exon) in rat and human species were analyzed, and found that several putative binding sites of c-Ets transcription factor could be identified (Supplementary Figure 4). However, the exact locations of these c-Ets sites and their functions may need further analysis. These findings suggested that the downstream mechanisms of P2Y1R and P2Y2R in up-regulating the expressions of PRiMA and ChAT might be the same as that of AChE. In addition to these three cholinergic genes, the regulatory effect of P2Y2R on acetylcholine receptors should also be addressed. Our preliminary results showed that the treatment of UTP and PSB114 could increase the mRNA expression of muscarinic AChR type 1 and 3 (AChR M1 and AChR M3) in cortical neurons (Unpublished data).

As in neurons, P2Y2R activation induced ACHE gene expression in muscle cells (Tung et al., 2004), but the AChE G4 isoform and AChE enzymatic activities were not changed upon treatment with UTP and other P2Y2R specific agonists. This suggests that ATP-, UTP- and P2Y2R-induced protein expression contribute to an inactive pool of AChE in both neurons (Supplementary Figure 2) and muscle cells (Tung et al., 2004). Active and inactive pools of AChE occur in native myotubes, wherein around 80% of the AChE protein is in the inactive pool (Rotundo RL, 1988). In this study other cholinergic genes (ChAT and PRiMA) were also up-regulated upon P2Y2R activation. Our data further showed that the treatment of cultured cortical neurons with UTP and P2Y2R-specific agonists tended to increase the protein level of AChE slightly more than that of PRiMA, raising the
possibility that PRiMA in neurons is insufficient for the complete assembly and stabilization of the final multi-meric form of functional AChE (Perrier et al., 2002).

In conclusion, ATP has been suggested to play a crucial role in signal transduction and in the post-synaptic gene regulation. In this study, P2Y$_2$R was shown to be expressed together with ATP-sensitive P2Y$_1$R in cultured cortical neurons. Activation of P2Y$_2$R by its agonist UTP triggered a MAP kinase-dependent signaling pathway that led to elevated expression of synaptic genes, such as the cholinergic genes encoding AChE, ChAT and PRiMA. Significantly, the actions of P2Y$_1$R and P2Y$_2$R in cortical neurons were found to be synergistic, and our results thus provide evidence for the co-existence of (at least) P2Y$_1$R and P2Y$_2$R at synapses where these receptors may together mediate the trophic effects of ATP and UTP.

**Authorship Contributions**

*Participated in research design:* Choi, Barnard, Tsim

*Conducted experiments:* Choi, Chu, A.W. Yung, L.Y. Yung

*Contributed new reagents or analytic tools:* Simon, Barnard

*Performed data analysis:* Choi, Chu, Siow, Lee, Lo, Dong

*Wrote or contributed to the writing of the manuscript:* Choi, Barnard, Tsim
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Footnotes

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Legends for Figures

FIGURE 1. Up-regulation of P2Y2R in rat brain from embryonic to adult stages. [A]: Cerebral cortex tissues from embryonic day 18 (E18) and adult rats were collected for mRNA extraction and reverse transcription (RT)-PCR analysis. Examination of PCR products on gels revealed the presence of transcripts encoding P2Y1R (~318 bp), P2Y2R (~200 bp), P2Y4R (~550 bp) and P2Y6R (~418 BP). Rat brain genomic DNA was used as a positive control and representative gel images are shown. [B]: Real-time PCR was used for precise quantification of mRNA expression. Data are normalized and expressed relative to the basal reading for E18 cortex (set at 1); mean ± SEM, n = 4 (n refers to number of independent experiments here and below). [C]: HEK293T cells transfected with HX-tagged P2Y2R-encoding cDNA or empty vector were triple stained by TO-PRO-3 (for cell nuclei), anti-P2Y2R and/or anti-Xpress antibodies (see Methods) and examined by confocal microscopy. In blocking experiments, the antigenic peptide from P2Y2R was pre-incubated with anti-P2Y2R before immuno-staining. Representative images shown; n = 4, scale bar = 10 μm. [D]: Western blotting confirmed that anti-P2Y2R stained an ~65 kDa protein band corresponding to P2Y2R in transfected HEK293T cells; this staining was also blocked by the pre-incubation of antibody with the antigenic peptide. [E]: Western blotting demonstrated the expression of P2Y2R protein in E18 and adult rat cortex tissues. GAPDH (~40 kDa) and α-tubulin (~55 kDa) were used as controls that indicate equal protein loading. Representative images shown, n = 4.

FIGURE 2. Differentiation profile of P2Y2R in rat cortical neurons. [A]: To demonstrate the
homogeneity of cortical neuron preparations, cultures were grown to 15 DIV with or without Ara-C (to prevent the proliferation of glia), stained by anti-GFAP (glial marker) and anti-MAP-2 (neuronal marker) antibodies, and examined by confocal microscopy. Representative photos shown; n = 4, scale bar = 40 μm. [B]: Total RNA isolated from cultured cortical neurons at 15 DIV was subjected to RT-PCR to reveal the presence of P2Y$_2$R mRNA. Rat genomic DNA was used as a positive control, while mRNA without reverse transcriptase (-RT) served as a negative control. Representative images shown, n = 4. [C]: The expression profile of P2Y$_2$R during the differentiation of cultured cortical neurons (5-25 DIV) was determined by Western blotting. The level of P2Y$_2$R protein (~65 kDa) was dramatically increased during the differentiation process. The expression of two differentiation markers, PSD-95 (~95 kDa) and NF-200 (~200 kDa), was also increased. GAPDH protein was used as a loading control and band intensities were quantified by calibrated densitometry. Data are normalized and expressed relative to the reading at 5 DIV, which was set at 1; mean ± SEM, n = 4.

**FIGURE 3.** Post-synaptic localization of P2Y$_2$R in cultured cortical neurons. Three-week-old cultured cortical neurons were fixed and double-stained with anti-P2Y$_2$R (green) and anti-PSD-95 (red) antibodies and examined by confocal microscopy. The smaller lower panels in this figure show higher magnification images of the boxed region in the ‘merged’ image (upper right). Images were superimposed to assess the extent of co-localization between P2Y$_2$R and PSD-95 (yellow). Representative images shown; n = 6, scale bars = 20 μm.
FIGURE 4. Association of P2Y2R with membrane rafts in adult brain and cortical neurons. [A]: Membrane lipid-rafts were prepared from adult rat cortex or cultured neurons using a discontinuous sucrose density gradient. Fractions were analyzed by Western blotting to determine the association of P2Y1R or P2Y2R with membrane rafts (fractions 5–7). Flotillin-2 was used a raft-marker, while AChE, mAChRM1, NR1, NR2A and PSD-95 served as markers for synaptic proteins in membrane rafts. Lysates from cerebral cortex and cortical neurons were used as positive controls for immunoblotting; representative images shown, n = 4. [B]: The band-intensities of P2Y1R and P2Y2R in membrane raft fractions were quantified by calibrated densitometry. Data are expressed as the percentages of receptor in lipid raft fractions relative to the total (i.e., lipid raft fractions + non-raft fractions); mean ± SEM, n = 4.

FIGURE 5. Stimulation of cholinergic gene expression by P2Y2R in cortical neurons. [A]: To demonstrate agonist specificity, HEK293T cells were transfected with pcDNA3 (mock control), P2Y1R or P2Y2R cDNAs, and then labeled with Fluo 4-AM to monitor Ca2+ mobilization. The general agonists used were ATP (100 nM) and UTP (100 nM) and the receptor-specific agonists were MRS2365 (100 nM) for P2Y1R, and 2SUTP (100 nM), PSB1114 (100 nM) and MRS2768 (100 nM) for P2Y2R. [B]: Cortical neurons at 14 DIV were treated with different agonists as in [A] with concentration to be 10 μM each and TPA (100 nM; positive control) for 15 hours. Total RNA was extracted to quantify the change in mRNA expression (by real-time PCR) for the cholinergic genes AChE, ChAT and PRiMA. [C]: Cultured neurons were exposed for 48 hours to drugs as in [B] and extracts prepared from them were examined by Western blotting. As these representative blots
show, antibody staining revealed the expression of AChE (≈68 kDa), ChAT (≈65 kDa) and PRiMA (≈20 kDa). Treatment with TPA (10 nM) served as a positive control, and GAPDH (≈40 kDa) was used as a loading control. Band intensities were quantified by calibrated densitometry, and the data are normalized and expressed relative to the basal reading (buffer-treated control set as 1); mean ± SEM, n = 4.

**FIGURE 6.** Participation of Ca^{2+} and Erk1/2 signaling in mediating P2Y_{2}R-induced cholinergic gene expression in cortical neurons. [A]: Cortical neurons at 14 DIV were labeled with Fluo 4-AM and then challenged with UTP (10 μM) or PSB1114 (10 μM) before Ca^{2+} measurement. In the representative traces shown here, the arrow indicates the time of drug treatment. The calcium ionophore A23187 (10 μM) was used as a positive control. [B]: Cortical neuron cultures (14 DIV) were serum starved and exposed to agonists (10 μM UTP and 10 μM PSB1114) and TPA (100 nM) for 1 hour. Protein extracts were prepared and stained with antibodies against total ERK1/2 (≈42/44 kDa) and phospho-ERK (≈42/44 kDa) to monitor ERK1/2 phosphorylation levels. TPA served as a positive control for phosphorylation, and protein band intensities were determined by calibrated densitometry. [C]: Cortical neurons (14 DIV) were treated for 1 hour with BAPTA-AM (calcium chelator; 5 μM), Gö6976 (PKC inhibitor; 1 μM) or PD98059 (MEK1 inhibitor; 20 μM) before adding PSB1114 (10 μM) for 5 and 10 min. Western blotting of extracts from these cultures revealed the extent of ERK1/2 phosphorylation. [D]: Cultures pre-treated with blockers as in [C] were challenged with UTP (10 μM) or PSB1114 (10 μM) for 15 hours. Total mRNAs were extracted and subjected to real-time PCR analysis to quantify the expression of AChE, ChAT and...
PRIiMA mRNAs. Data are normalized and expressed as ratios relative to the basal reading (DMSO/H2O-treated control set as 1); mean ± SEM, n = 4.

FIGURE 7. Synergistic effect of P2Y1R and P2Y2R in activating cholinergic gene expression. [A]: Cortical neurons at 14 DIV were labeled with Fluo 4-AM and then treated with P2Y1R agonist alone (10 μM MRS2365), P2Y2R agonist alone (10 μM PSB1114), or the two receptor agonists combined (PSB1114 + MRS2365) before Ca2+ measurement. The arrow indicates the time of agonist addition. [B]: Neurons at 14 DIV were treated with agonists as in [A], or additionally with MRS2768 (another P2Y2R-specific agonist) or MRS2768 + MRS2365, for 15 hours. Total mRNAs were extracted and the expression of cholinergic genes AChEβ, ChAT and PRIiMA was analyzed by quantitative real-time PCR. [C]: Neurons were exposed to agonists as in [A] for 48 hours and extracts were collected for Western blotting. Co-activation of P2Y1R and P2Y2R led to higher expression of AChEβ, ChAT and PRIiMA proteins than the stimulation of just one of the two receptors. Data are normalized and expressed relative to the basal reading with H2O-treated control (set as 1); mean ± SEM, n = 4.
Figure 1
Figure 2

(A) Immunofluorescence images showing GFAP and MAP-2 staining in cortical neurons treated with Ara-C with or without + Ara-C. The images illustrate the effects of Ara-C on astrocyte and neuronal markers.

(B) RT-PCR analysis of P2Y2R expression in cortical neurons. The RT-PCR bands are 200 bp in size, indicating the presence of P2Y2R mRNA.

(C) Western blot analysis of P2Y2R, PSD-95, and NF-200 expression during differentiation. The graph shows the amount of P2Y2R expression (x Basal) over the course of differentiation (DIV), with increases at 10, 15, 20, and 25 DIV.
Figure 4
Figure 5
Figure 6
Figure 7