Activation of UTP-Sensitive P2Y₂ Receptor Induces the Expression of Cholinergic Genes in Cultured Cortical Neurons: A Signaling Cascade Triggered by Ca²⁺ Mobilization and Extracellular Regulated Kinase Phosphorylation


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ABSTRACT

ATP functions as an extracellular signaling molecule that is costored and coreleased with neurotransmitters at central and peripheral neuronal synapses. Stimulation by ATP upregulates 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 tropic 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 colocalized with the postsynaptic protein PSD-95 in cortical neurons. Notably, agonist-dependent stimulation of P2Y₂R elicited 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 extracellular regulated kinases 1/2 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 demonstrated the influence of costimulation 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 (P2Y₁,11,12,13Rs), uracil nucleotides UTP and UDP (P2Y₂,4,6Rs), and UDP-glucose (P2Y₁₄R). In the central nervous system (CNS), P2Y₁₂,4,6Rs have been detected in both neuronal and glial cells (Hussel and Boehm, 2006). The P2YRs are G protein-coupled receptors that activate phospholipase C (PLC) via Gα₁/11 and stimulate or inhibit adenyl cyclase via Gα₅ and Gα₁₁. 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 neurologic functions in both the CNS and the peripheral nervous system. Most of the studies on ATP signaling have focused on ATP/P2YRs. At the
neuromuscular junction (NMJ), for example, ATP is coreleased with acetylcholine (ACh) and acts as a neuromodulator or neurotransmitter on postsynaptic muscle cells (Tsim and Barnard, 2002). ATP together with a neuropeptide, calcitonin gene-related peptide, can potentiate the postsynaptic ACh receptor (AChR) channel activity in the early phase of neuromuscular synaptogenesis in Xenopus (Fu, 1995). Studies from our laboratory indicated that ATP, via P2Y2R and P2Y2R, plays a critical role in regulating gene transcription and in forming postsynaptic specializations at the NMJ (Choi et al., 2001, 2003; Ling et al., 2004; Tung et al., 2004). Moreover, the activation of P2YR triggered a mitogen-activated protein kinase signaling cascade, which subsequently upregulated 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.

UTP is another vital biomolecule that elicits significant physiologic responses in epithelial cell ion transport, hormone release, and synaptic transmission (Lazarowski and Boucher, 2001). In mammalian cells UTP is a natural ligand for P2Y2R and P2Y2R (Burnstock, 2007), which cannot be easily distinguished pharmacologically because both are activated by UTP. Previously, UTP and catecholamine were found in molar ratio of approximately 0.1:5 in chromaffin granules (Zimmermann, 1994), and a ratio of 1:3–5 was observed for extracellular UTP/ATP in both resting and mechanically stimulated cell lines (Lazarowski et al., 2003). P2Y2R mediates neuronal differentiation via nerve growth factor/TrkA signaling (Arthur et al., 2005), performs neuroprotective, antiapoptotic or proapoptotic functions, and modulates glutamate N-methyl-d-aspartate (NMDA) receptor currents or intracellular Ca2+ (Kim et al., 2003; Cavaliere et al., 2005; Winkler et al., 2007). Although many different lines of evidence have now demonstrated the physiologic functions of UTP in diverse systems, the regulatory effects of the UTP-sensitive P2Y2R on neuronal gene expression remain largely unknown. Here, we present results of studies on the expression and cellular localization of P2Y2R in rat brain and cortical neurons and the influence of the receptor on the expression of synaptic genes.

Materials and Methods

Materials. Sprague-Dawley rats were provided by the Animal Care Facility at the Hong Kong University of Science and Technology. Cell culture media were purchased from Invitrogen (Carlsbad, CA) and commercial antibodies were from Sigma-Aldrich (St. Louis, MO), except where specifically noted. Appryase (Grade VII) and phorbol ester (TPA) were from Sigma-Aldrich. To ensure the purity of UTP and ATP, stock solutions (10 mM; Tocris Bioscience, Bristol, UK) were pretreated in buffer with 20 U/ml creatine phosphokinase and 10 mM ATP, molar ratio of approximately 0.1:5 in chromaffin granules and commercial antibodies were from Sigma-Aldrich (St. Louis, MO), Cell culture media were purchased from Invitrogen (Carlsbad, CA) and media and buffer were from BioWhittaker, Belgium, except where specified otherwise. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s modified Eagle's medium 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 appryase (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 15–24 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 ultraviolet absorbance at 260 nm, and 5 μg of the RNA from cortex and neurons was reverse-transcribed by Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) in the presence of oligo(dT) primers. Total cDNA from the cortex or cultured cortical neurons (500 ng each) was amplified by polymerase chain reaction (PCR) using Taq DNA polymerase (1:25 U/reaction) in a reaction mixture containing these primers: P2Y2R: 5’-CTC TAC TTT GTC ACC ACC AGC GC-3’ (forward) and 5’-GGT GAT GTG-3’ (reverse); P2Y4R: 5’-GCC ATT AGA CAC CTT GTA-3’ (forward) and 5’-AAAG CCA AGC AGA CAA CCA-3’ (reverse); and P2Y1R: 5’-CGC TTC TCC TTC TCC GAT AA-3’ (forward) and 5’-GTA GGG TCT CTT GAT GTA TCT-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 transcription. Amplifications were performed in a GeneAmp 9700 PCR System (Applied Biosystems, Foster City, CA) for 40 cycles (94°C/45 seconds; annealing 58°C/45 seconds; 72°C/1 minute). 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’-GGT GAT GTG-3’ (reverse); AChET catalytic subunit: 5’-GTG CAA AGA GCC ACC TGA G-3’ (forward) and 5’-TGA CAG GTG TCA GCA GGG TTC CTG-3’ (reverse) (Boudreau-Lariviére et al., 2000); ChAT: 5’-GGT TTA AAG CAA GCC ACC TCC G-3’ (forward) and 5’-GGT GGG TCG GGA TCG TTC AGC CCC-3’ (forward) and 5’-TCA CAG GTG TCA GCA GGG TTC CTG-3’ (reverse) (Boudreau-Lariviére et al., 2000); ChAT: 5’-GGT TTA AAG CAA GCC ACC TCC G-3’ (forward) and 5’-GGT GGG TCG GGA TCG TTC AGC CCC-3’ (forward) and 5’-TCA CAG GTG TCA GCA GGG TTC CTG-3’ (reverse) (Boudreau-Lariviére et al., 2000); proline-rich membrane anchor (PRMA): 5’-CTC TACT GCT GTG AGC AGC GTT CAC-3’ (forward) and 5’-CTG TTC ACC AGG AGA TCC CAG AGG-3’ (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5’-AAC GGA TTT GGC GGT ATT GTG CAC-3’ (forward) and 5’-CTT CCC GTT CAG CTC TGG G-3’ (reverse) (Lee et al., 2004). SYBR green signal was detected on the Mx3000P 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 subcloned into pcDNA His-Max expression vector using Pfu (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 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 electrophoresed 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; AP-010; Alomone Laboratories, Jerusalem, Israel), anti-AChE (1:500; Clone E19; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-ChAT (1:2000; Millipore, Billerica, MA), anti-PSD-95 (1:1500; Millipore), anti-neurofilament 200 (NF-200; 1:2000; Sigma-Aldrich), anti-flotillin-2 (1:1000; BD Biosciences, Franklin Lakes, NJ), anti-P2YR1 (1:1000; self-generated; 24), anti-mAChR M1 (1:1000; Sigma-Aldrich), anti-phospho-extracellular regulated kinases (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.

Immunofluorescent Staining. Cultured cortical neurons or HEK293T cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes. After being washed with 50 mM ammonium chloride (NH₄Cl) for 25 minutes, cultures were permeabilized by 0.1% Triton X-100 in PBS for 10 minutes and then blocked with 5% bovine serum albumin 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; AP-010; Alomone Laboratories), anti-Xpress (1:1000; Invitrogen), Cy3-conjugated anti-GFAP (1:2000; Sigma-Aldrich), anti-MAP-2 (1:500; Sigma-Aldrich), and anti-PSD-95 (1:500; Millipore). After PBS washes (3 × 15 minutes), cultures were labeled with Alexa 488- or 555-conjugated secondary antibodies (Invitrogen) plus the nuclear stain DAPI (1:10,000) for 1 hour at room temperature. After washing with three more times with PBS (15 minutes each), samples were dehydrated serially in ethanol and mounted with fluorescent mounting medium (Dako, Carpinteria, CA). Confocal fluorescence microscopy (Zeiss LSM 510; Carl Zeiss AG, Oberkochen, Germany) 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 pseudocolor (TO-PRO-3).

Membrane Raft Preparation. Membrane rafts were prepared as previously described (Xie et al., 2010). 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 seconds 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 second with 30-second intervals to avoid heating, and the samples were centrifuged at 500g for 5 minutes to remove cell debris and nuclei. The postnuclear extracts of tissues or cells were centrifuged at 180,000g for 30 minutes in a Sorvall TST 60.4 rotor, and the resulting pellet was washed and resuspended by sonication each 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 20 fractions (190 μl each) were collected from the top and used for Western blotting.

Fluorometric Measurement of Ca²⁺ 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 Hanks’ balanced salt solution. Cortical neurons (15 DIV) were also labeled by the same procedure, except that tetrodotoxin (100 nM; Sigma-Aldrich) was added to the buffer to obtain a stable baseline reading. All agonists were prepared in Hanks’ balanced salt solution, 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 (GraphPad Software, La Jolla, CA).

Data Analysis. Gel documentation and relative quantification were performed with the IS1000 Digital Imaging System. To obtain colocalization ratios of proteins in cultured neurons, 20 neurons that appeared morphologically healthy in phase contrast were randomly selected from each double-immunolabeled coverslip; all immunopositive puncta present in 50-μm dendrite segments were counted, with only puncta larger than 10 pixels being used to avoid including the background staining. The colocalization ratio (%) for each pair of immunopositive puncta was calculated thus: (number of puncta double-immunostained/number of total puncta immunostained with one of the two antibodies) × 100. On average 80 puncta were analyzed per neuron and statistical analyses were by two-tailed, unequal, one-way analysis of variance. Data are plotted as mean ± S.E.M. (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 four 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 reverse-transcription polymerase chain reaction (RT-PCR) analysis (Fig. 1A). P2Y1R, P2Y2R, 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 specificity of the antibody, P2Y2R was tagged with Xpress epitope and transfected into HEK293T cells for double immunolabeling 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 preincubation of the probing solution with the antigenic peptide from P2Y$_2$R blocked the fluorescent signal from anti-P2Y$_2$R but not anti-Xpress antibody (Fig. 1C). In Western blotting, P2Y$_2$R protein was detected as an $\sim 65$-kDa band in extracts of P2Y$_2$R-transfected HEK293T cells, and this staining was also abolished when anti-P2Y$_2$R antibody was preincubated with blocking peptide (Fig. 1D). These results indicated that the anti-P2Y$_2$R antibody specifically recognized its target. Anti-P2Y$_2$R also stained P2Y$_2$R in rat brain lysates, where the expression of the protein increased from embryonic to adult stages (Fig. 1E); here $\alpha$-tubulin and GAPDH served as loading controls. The receptor size of $\sim 65$ 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 P2Y$_2$R was present in cortical neurons and upregulated during neuronal differentiation.

Although P2Y$_2$R levels increased during brain development (above), this upregulation could have occurred in both glia and neurons. To specifically examine neuronal P2Y$_2$R, primary cortical neurons were used as a model (Brewer, 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 P2Y$_2$R and P2Y$_4$R (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, whereas 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 P2Y$_2$R 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-P2Y$_2$R next revealed the expression profile of P2Y$_2$R protein during the differentiation of cortical neurons (5 to 25 DIV): the P2Y$_2$R protein level increased throughout this period (Fig. 2C, upper panel). The proper differentiation of these neuronal cultures was confirmed by the parallel upregulation of two markers, NF-200

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**Fig. 1.** Up-regulation of P2Y$_2$R 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 RT-PCR analysis. Examination of PCR products on gels revealed the presence of transcripts encoding P2Y$_1$R ($\sim 318$ bp), P2Y$_2$R ($\sim 200$ bp), P2Y$_4$R ($\sim 550$ bp) and P2Y$_6$R ($\sim 418$ 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 $\pm$ S.E.M., $n = 4$ ($n$ refers to number of independent experiments here and below). (C) HEK293T cells transfected with HX-tagged P2Y$_2$R-encoding cDNA or empty vector were triple stained by TO-PRO-3 (for cell nuclei), anti-P2Y$_2$R and/or anti-Xpress antibodies (see Materials and Methods) and examined by confocal microscopy. In blocking experiments, the antigenic peptide from P2Y$_2$R was pre-incubated with anti-P2Y$_2$R before immuno-staining. Representative images shown; $n = 4$, scale bar = 10 $\mu$m. (D) Western blotting confirmed that anti-P2Y$_2$R stained an $\sim 65$ kDa protein band corresponding to P2Y$_2$R 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 P2Y$_2$R protein in E18 and adult rat cortex tissues. GAPDH ($\sim 40$ kDa) and $\alpha$-tubulin ($\sim 55$ kDa) were used as controls that indicate equal protein loading. Representative images shown, $n = 4$. 

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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 images shown, *n*=4. (B) Total RNA isolated from cultured cortical neurons at 15 DIV was subjected to RT-PCR to reveal the presence of P2Y2R mRNA. Rat genomic DNA was used as a positive 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 ± S.E.M., *n*=4. (C) The expression profile of P2Y2R during the differentiation of cultured cortical neurons (5-25 DIV) was determined by Western blotting. The level of P2Y2R 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 ± S.E.M., *n*=4.

**Fig. 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 P2Y2R 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 P2Y2R during the differentiation of cultured cortical neurons (5-25 DIV) was determined by Western blotting. The level of P2Y2R 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 ± S.E.M., *n*=4.

Cellular Localization of P2Y2R. To investigate the cellular distribution of P2Y2R, cortical neurons were labeled with the anti-P2Y2R antibody. The resulting immunofluorescent staining revealed a synaptic localization of P2Y2R (Fig. 3): in mature cortical neurons, P2Y2R (green) were found on the cell body but were also enriched in the dendrites, where they partially colocalized with the postsynaptic marker PSD-95 (red). Quantification of this colocalization 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; 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 P2Y2R and/or P2Y2R was associated with membrane rafts. This restriction localization of the two receptors in this microdomain would provide spatial advantage in mediating the synergetic action that was revealed in the rest of the study. Extracts of adult cortex and cortical neurons (30 DIV) were prepared and separated by a discontinuous sucrose density gradient to isolate membrane rafts. P2Y2R (~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 immunoblots total lysates were used as positive controls. In addition to P2Y2R 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 postsynaptic protein PSD-95 (~95 kDa) (Wong and Schlichter, 2004). Densitometry indicated that ~45% of P2Y2R in adult cerebral cortex was associated with membrane rafts, whereas ~86% of P2Y2R 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 P2Y2R and P2Y2R in neurons and adult brain and suggested a potential role of the UTP-responsive P2Y2R receptors, such as 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 control cells transfected with pcDNA3 (mock), addition of ATP and UTP slightly

(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- and ~11-fold, respectively and that the expression of P2Y2R went up ~7-fold during the differentiation process (Fig. 2C, lower panel).

**Fig. 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 P2Y2R mRNA. Rat genomic DNA was used as a positive 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 ± S.E.M., *n*=4.

**Fig. 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 P2Y2R mRNA. Rat genomic DNA was used as a positive 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 ± S.E.M., *n*=4.
induced Ca\(^ {2+}\) elevation, suggesting that low levels of endogenous P2Y receptors were present in HEK293T cells (Fig. 5A). After the overexpression of P2Y2R, the Ca\(^ {2+}\) signals elicited by ATP and UTP were both higher than that seen in the mock-transfected controls (as expected), and with the addition of P2Y2R-specific agonists (2SUTP, PSB1114, and MRS2768) these responses were even higher (Fig. 5A). In other controls, the P2Y1R-specific agonist MRS2365 did not trigger Ca\(^ {2+}\) elevation in mock- or P2Y2R-transfected cultures, and the P2Y2R-specific agonists failed to elicit a response in P2Y1R-transfected cultures (Fig. 5A). In other controls, the P2Y2R-specific agonist MRS2365 did not trigger Ca\(^ {2+}\) elevation in mock- or P2Y2R-transfected cultures, and the P2Y2R-specific agonists failed to elicit a response in P2Y1R-transfected cultures (Fig. 5A). The dose-response curves of these agonists (MRS2365 for activating P2Y1R, 2SUTP, PSB1114, and MRS3768 for activating P2Y2R) are shown in Supplemental Fig. 1. On the basis of these pharmacological studies, the P2Y2R-specific agonists 2SUTP, PSB1114, and MRS2768 were employed for the specific activation of P2Y2R 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 P2Y2R-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- to 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 ATP or P2Y2R-specific agonists, the protein levels of AChE, ChAT, and PRiMA were upregulated to different extents (Fig. 5C). ATP and TPA were used as positive controls, and GAPDH was immunoblotted as a normalization control for protein quantification. Because P2Y2R activation increased the AChE protein expression, it was of interest 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 (Supplemental Fig. 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, of which five of them act through Gq-coupled signaling cascade. Activation of P2Y2R can stimulate PLC and increase Ca\(^ {2+}\) 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 "classic" signaling was initiated by P2Y2R in cortical neurons. Cortical neurons were preloaded with the Ca\(^ {2+}\) indicator Fluo 4-AM, and upon the addition of UTP or PSB1114 the intracellular Ca\(^ {2+}\) was found to be sharply elevated relative to controls (Fig. 6A).
For this response the Ca$^{2+}$ ionophore A23187 served as a positive control.

The activation of P2Y$_2$R in cultured cortical neurons also led to the phosphorylation of extracellular regulated kinases 1 and 2 (ERK1, $\sim$44 kDa; ERK2, $\sim$42 kDa), which was shown by Western blotting (Fig. 6B): treatment with UTP or PSB1114 induced the phosphorylation of ERK1/2, with the peak response occurring at 5 minutes and then gradually declining until 60 minutes. TPA served as a positive control for the stimulation of ERK1/2 phosphorylation. The above results suggested that the activation of P2Y$_2$R can trigger Ca$^{2+}$ mobilization and induce ERK1/2 phosphorylation in cultured cortical neurons, which is consistent with previous findings with P2Y$_2$R in muscle and other cell types.

Lastly, to ascertain if Ca$^{2+}$ and MAP kinase-dependent pathways participate downstream from P2Y$_2$R in regulating the expression of synaptic genes in cortical neurons, we used the pharmacological inhibitors BAPTA-AM [1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)] (Ca$^{2+}$ chelator), Gö6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-al]pyrrolo[3,4-c]carbazole] (PKC inhibitor), and PD98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one] (MEK1 inhibitor). To confirm the effectiveness of these blockers, cultured cortical neurons were treated with the inhibitors for 1 hour before activating P2Y$_2$R 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 pretreatment did not affect the basal expression of AChE, ChAT, or PRiMA mRNAs (Fig. 6D), but the inhibitors abolished the upregulation 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 Ca2+- 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 coexisting in cortical neurons. The coexistence of P2Y1R and P2Y2R in the same postsynaptic terminal were partially supported by our previous and current findings of double immunostaining of PSD-95 with P2Y1R (Siow et al., 2010) and PSD-95 with P2Y2R (Fig. 3). Given their distinct ligand selectivity (ATP/ADP for P2Y1R and UTP for P2Y2R) as well as their synaptic localization in neurons, we hypothesized...
a synergistic action between P2Y1R and P2Y2R. To test this, cortical neurons were cotreated with the P2Y1R-specific agonist MRS2365 and the P2Y2R-specific agonists PSB1114 and MRS2768 and then analyzed for Ca2+ mobilization and gene expression. A synergy in P2Y1R and P2Y2R actions was first demonstrated by a robust increase of intracellular Ca2+ (Fig. 7A): the activation of P2Y1R alone (by MRS2365) or P2Y2R alone (by PSB1114) caused an ~2-fold increase in Ca2+ level relative to control, whereas the coapplication of the two receptor agonists produced an ~5-fold elevation of Ca2+. Next, at the gene expression level, the mRNAs encoding AChE, ChAT, and PRiMA increased ~4-fold after P2Y1R activation, which was similar to that seen with the stimulation of P2Y2R (Fig. 7B). However, treatment with MRS2365 + PSB1114 (or MRS2365 + MRS2768) induced gene expression ~8-fold (Fig. 7B). The coactivation of P2Y1R and P2Y2R (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 coactivation of two P2Y receptors under physiologic conditions (with ATP or ADP for P2Y1R, and UTP for P2Y2R) 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 postsynaptic 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 peripheral nervous system and CNS (Kunapuli and Daniel, 1998; Dubyak, 2003; Jørgensen, 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 P2Y<sub>2</sub>R 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 P2Y<sub>2</sub>R 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 immunofluorescent staining P2Y<sub>2</sub>R was localized at postsynaptic regions. Postsynaptic P2Y<sub>2</sub>Rs are thought to be responsible for initiating signal transmission, whereas the presynaptic receptors are primarily involved in modulating neurotransmitter release (Cunha and Ribeiro, 2000). We also found that both P2Y<sub>1</sub>R and P2Y<sub>2</sub>R were concentrated in membrane lipid rafts, as were several other postsynaptic 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 P2Y<sub>2</sub>R may imply a postsynaptic signaling function in neurons, which might participate in the coregulation of AChE and NR2A by P2Y<sub>1</sub>R (Siow et al., 2010). More importantly, the coexistence of P2Y<sub>1</sub>R and P2Y<sub>2</sub>R 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 physiologic conditions, ATP is released in response to myriad stimuli (Novak, 2003). ATP is a cotransmitter in synaptic vesicles together with neurotransmitters and also is 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 nanomolar 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 ectonucleotidases that degrade extracellular ATP and UTP to ADP and UDP, respectively. Nevertheless, whether UTP is released from neurons or glia, the presence of P2Y<sub>2</sub>R in cortical neurons indicates the importance of signaling by this receptor of UTP in neurons. One interesting issue is that ATP is the agonist for both P2Y<sub>1</sub>R and P2Y<sub>2</sub>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. 5, B and C, were rather similar. This might imply that the coactivation of two receptors by ATP could not provide the maximum efficacy of receptor activation for synergistic effect, as the drug potency of P2Y<sub>2</sub>R is MRS2365 > 2-MeSADP > ADP > ATP, whereas that of P2Y<sub>2</sub>R is PSB1114 > MRS2768 > UTP ∼ ATP.

**MAP Kinase-Dependent Signaling Pathway Induced by P2Y<sub>2</sub>R Activation.** The signaling pathway of P2Y<sub>2</sub>R is well-documented (Burnstock, 2007). P2Y<sub>2</sub>R couples to PLC-β via Go<sub>q</sub> to mediate IP<sub>3</sub> production, which results in the release of intracellular Ca<sup>2+</sup> and activation of PKC. Treatment of UTP (P2Y<sub>2</sub>R and P2Y<sub>2</sub>R agonist) and PSB1114 (P2Y<sub>2</sub>R-specific agonist) boosted intracellular Ca<sup>2+</sup> mobilization. To further elaborate the UTP-induced signaling, we investigated ERK phosphorylation. MAP kinases are a group of serine-threonine kinases that transduce the external signals into intracellular biologic events. The MAP kinase-dependent pathway involving Raf/MEK/ERK responds to the activation of tyrosine kinase receptors and also to P2Y<sub>2</sub>R or P2Y<sub>2</sub>R signaling in muscle cells and in P2Y<sub>2</sub>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 (P2Y<sub>2</sub>R-specific agonists) induced robust phosphorylation of ERK1 and ERK2 in a time-dependent manner. Our results suggest that MAP kinase signaling is activated by Ca<sup>2+</sup> and PKC, and it appears that nucleotide-mediated P2Y<sub>2</sub>R 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 Ca<sup>2+</sup> mobilization and Erk phosphorylation in Fig. 6 were higher than that of P2Y<sub>2</sub>R-specific agonist PSB1114. This might be attributed to the simultaneous activation of P2Y<sub>2</sub>R by UTP in the cultures. From our preliminary results, P2Y<sub>2</sub>R also existed in mature cortical neurons, because activation of P2Y<sub>2</sub>R could also trigger IP<sub>3</sub> formation and Ca<sup>2+</sup> mobilization via Go<sub>q</sub> coupling (unpublished data). This was a critical reason for using the P2Y<sub>2</sub>R-specific agonists for testing rather than UTP in the current study.

Activation of P2Y<sub>2</sub>R 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 P2Y<sub>2</sub>R via Raf/MEK/ERK pathway in muscles (Choi et al., 2003) and in cortical neurons (Siow et al., 2010). For P2Y<sub>2</sub>R, the similar regulatory pathway was also reported in muscles (Tung et al., 2004) and in cortical neurons (Supplemental Fig. 3). By use of an AChE promoter tagged with a luciferase reporter, the treatment of UTP and different P2Y<sub>2</sub>R 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 (Etk-1) partially abolished the inductive effects of those agonists (Supplemental Fig. 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 (∼3 kb 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 (Supplemental Fig. 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 P2Y<sub>2</sub>R and P2Y<sub>2</sub>R 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 (AChRM1 and AChRM3) 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 (Supplemental Fig. 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 (Rotondo, 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, P2Y2R was shown to be expressed together with ATP-sensitive P2Y2R in cultured cortical neurons. Activation of P2Y2R 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 P2Y2R and P2Y2R in cortical neurons were found to be synergistic, and our results thus provide evidence for the co-existence of (at least) P2Y2R and P2Y2R 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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Supplementary Figure 1

Dose-response curves of MRS2365, PSB1114 and MRS2768 in inducing Ca\(^{2+}\) mobilization in transfected HEK293T cells

HEK293 cells were transiently transfected with P2Y\(_1\)R or P2Y\(_2\)R cDNA by calcium phosphate precipitation method. Afterward, cultures were seeded on 96-well plates, and challenged with different concentrations of agonists (0, 0.1, 1, 10, 100, 1000 and 10000 nM) for Ca\(^{2+}\) mobilization assay using Fluo A-AM as indicator. Results showed that the application of these agonists could increase the intracellular Ca\(^{2+}\) level in dose-dependent manners. In all cases, 100 nM of these agonists could achieve more than 80% of total responses.
Activation of P2Y$_2$R did not affect the enzymatic activity and molecular forms of AChE in cortical neurons

[A]: Cortical neuron at 14 DIV was treated with different drugs (ATP, UTP, 2SUT, PSB1114, MRS2768, all at 10 µM) for 48 hours, and collected for AChE enzymatic activity assay. Results showed that none of the drug treatment could alter the enzymatic activity.

[B]: Cortical neuron at 14 DIV was treated with P2Y$_2$R agonist (10 µM UTP and 10 µM PSB1114) for 48 hours. Proteins were extracted and subjected to sucrose density gradient analysis to reveal the molecular form of AChE. Results showed that both G$_{1/2}$ and G$_4$ AChE forms were expressed in cortical neurons. However, their amounts were not changed by the activation of P2Y$_2$R.
P2Y$_2$R-induced AChE promoter activity in cortical neurons is mediated by c-Ets transcription factor binding site

[A]: Human AChE promoter with and without the deletion of binding sites for c-Ets transcription factor (Elk-1) were subcloned in pGL4 luciferase-reporter (Siow et al., 2010).

[B]: Cortical neurons at 1 DIV were transfected with DNA of pAChE-Luc or pAChE$\Delta$Elk-1 [1,3] -Luc by Nucleofactor transfection kit. At 15 DIV, cultures were treated with different P2Y$_2$R agonists, including UTP, 2SUTP, PSB1114 and MRS2768 (10 $\mu$M each) for 48 hours. After collection, lysates were subjected to luciferase activity assay where the readings were normalized by the protein content (Siow et al., 2010). Results showed that the activation of P2Y$_2$R by all agonists could stimulate the transcriptional activity of AChE promoter (pAChE-Luc). Interestingly, the deletion of two Elk-1 site (c-Ets transcription factor binding sites) could partially abolish the stimulatory responses of all agonists, suggesting that c-Ets transcription factor binding sites on AChE promoter were involved in P2Y$_2$R-induced AChE expression in cortical neurons.
Putative c-Ets binding sites on PRiMA and ChAT promoters

The 3 kb DNA sequence upstream of exon 1 of *PRiMA* and *ChAT* genes from human and rat species were subjected to TFSearch program to identify the putative binding sites of transcription factors ([http://www.cbrc.jp/research/db/TFSEARCH.html](http://www.cbrc.jp/research/db/TFSEARCH.html)). Results showed that in all the promoter regions, several putative c-Ets binding sites (▼) could be located.