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**Activation of UTP-sensitive $P2Y_2$ receptor induces the expression of cholinergic genes in cultured cortical neurons: A signaling cascade triggered by $Ca^{2+}$ mobilization and ERK phosphorylation**

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Running title: *Post-synaptic $P2Y_2$ receptor signaling in neurons*

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Abbreviation: Acetylcholinesterase, AChE; Central nervous system, CNS; Choline acetyltransferase, ChAT; Neuromuscular junction, NMJ; Peripheral nervous system, PNS; Proline-rich membrane anchor, PRiMA; P2Y receptor, P2YR;

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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 $P2Y_2R$ was the most abundant. $P2Y_2R$ 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 $P2Y_2R$ 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 $Ca^{2+}$ mobilization and ERK1/2 activation. The importance of $P2Y_2R$ action was further shown by the receptor's synergistic effect with $P2Y_1R$ in enhancing cholinergic gene expression via the robust stimulation of $Ca^{2+}$ influx. Taken together our results revealed a developmental function of $P2Y_2R$ in promoting synaptic gene expression and demonstrate the influence of co-stimulation of $P2Y_1R$ and $P2Y_2R$ in neurons.

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## 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 $G\alpha_{q/11}$, and stimulate or inhibit adenylyl cyclase via $G\alpha_s$ and $G\alpha_{i/o}$. 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 $P2Y_1R$ and $P2Y_2R$, plays a critical role in regulating gene transcription and in forming post-synaptic specializations at the NMJ (Choi et al.,

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2001; 2003; Ling et al., 2004, Tung et al., 2004). Moreover, the activation of $P2Y_1R$ 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 $P2Y_2R$ and $P2Y_4R$ (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). $P2Y_2R$ 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 $Ca^{2+}$ (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 $P2Y_2R$ on neuronal gene expression remain largely unknown. Here, we present results of studies on the expression and cellular localization of $P2Y_2R$ in rat brain and cortical neurons and the receptor's influence on the expression of synaptic genes.

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**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). $P2Y_1R$-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 $P2Y_2R$-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% $CO_2$ 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

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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% $CO_2$ 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: $P2Y_1R$: 5'-CCT GCG AAG TTA TTT CAT CTA-3' (forward) and 5'-GTT GAG ACT TGC TAG ACC TCT-3' (reverse); $P2Y_2R$: 5'-CTC TAC TTT GTC ACC ACC AGC GC-3' (forward) and 5'-GTG GTC CCA TAA GCC GGT TTG-3' (reverse); $P2Y_4R$: 5'-GGC ATT GTC AGA CAC CTT GTA-3' (forward) and '5-AAG GCA CGA AGC AGA CAG CAA-3' (reverse); and $P2Y_6R$: 5'-CGC TTC CTC 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

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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: $P2Y_2R$: 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 GCA GCG 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 $P2Y_2R$ cDNA was sub-cloned into pcDNA4 His-Max expression vector using *Pfx* (Invitrogen) to form N-terminal His-Xpress-$P2Y_2R$ (HX-$P2Y_2R$).

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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-$P2Y_2R$ (1:1000; APR-010; Alomone Labs, Israel), anti-$AChE_T$ (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

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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 ($NH_4Cl$) 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-$P2Y_2R$ (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 x15 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.,

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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 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

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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).

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## Results

*$P2Y_2R$ in brain and cultured cortical neurons*

To address the functional role(s) of $P2Y_2R$ 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**). $P2Y_1R$, $P2Y_2R$, $P2Y_4R$ and $P2Y_6R$ 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 $P2Y_2R$ mRNA showing the maximal change during development; expression of the $P2Y_2R$ transcript in adult was ~5-fold higher than at the E18 stage. Next, we examined the expression of $P2Y_2R$ protein with a commonly-used commercial anti-$P2Y_2R$ antibody, whose target epitope is located in the third intracellular loop of $P2Y_2R$. To test for the antibody's specificity, $P2Y_2R$ was tagged with Xpress epitope and transfected into HEK293T cells for double immuno-labeling with anti-$P2Y_2R$ and anti-Xpress antibodies. The overlap in fluorescent staining by these two antibodies indicated that anti-$P2Y_2R$ recognized the expressed $P2Y_2R$ in the transfected cells (**Fig. 1C**), and pre-incubation of the probing solution with the antigenic peptide from $P2Y_2R$ blocked the fluorescent signal from anti-$P2Y_2R$ but not anti-Xpress antibody (**Fig. 1C**). In Western blotting, $P2Y_2R$ protein was detected as an ~65 kDa band in extracts of $P2Y_2R$-transfected HEK293T cells, and this staining was also abolished when anti-$P2Y_2R$ antibody was pre-incubated with blocking peptide (**Fig. 1D**). These results indicated that the anti-$P2Y_2R$ antibody specifically recognized its target. Anti-$P2Y_2R$ also stained $P2Y_2R$ in rat brain lysates, where the protein's expression increased from embryonic to adult stages (**Fig. 1E**); here α-tubulin

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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 $P2Y_2R$ was present in cortical neurons and up-regulated during neuronal differentiation.

Although $P2Y_2R$ levels increased during brain development (above), this up-regulation could have occurred in both glia and neurons. To specifically examine neuronal $P2Y_2R$, 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 $P2Y_2R$ and $P2Y_4R$ (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 maker) 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 $P2Y_2R$ 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-$P2Y_2R$ next revealed the expression profile of $P2Y_2R$ during the differentiation of cortical neurons (5 DIV to 25 DIV): the $P2Y_2R$ 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

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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 $P2Y_2R$ went up ~7-fold during the differentiation process (**Fig. 2C lower panel**).

*Cellular localization of $P2Y_2R$*

To investigate the cellular distribution of $P2Y_2R$, cortical neurons were labeled with the anti-$P2Y_2R$ antibody. The resulting immuno-fluorescent staining revealed a synaptic localization of $P2Y_2R$ (**Fig. 3**): in mature cortical neurons, $P2Y_2Rs$ (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 $P2Y_2R$ 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 $P2Y_1R$ and/or $P2Y_2R$ 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

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and separated by a discontinuous sucrose density gradient to isolate membrane rafts. $P2Y_1R$ (~60 kDa) and $P2Y_2R$ (~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 $P2Y_1R$ and $P2Y_2R$, 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 $P2Y_1R$ in adult cerebral cortex was associated with membrane rafts, while ~86% of $P2Y_1R$ in cultured cortical neurons was in rafts (**Fig. 4B**). For $P2Y_2R$, 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 $P2Y_1R$ and $P2Y_2R$ in neurons and adult brain and suggested a potential role of the UTP-responsive $P2Y_2R$ receptors, like $P2Y_1R$, in regulating neuronal function(s).

*Regulation of cholinergic gene expression by $P2Y_2R$*

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 $P2Y_2R$-selective agonists. First, HEK293T cells were transfected with $P2Y_2R$ cDNAs to examine agonist specificity in the $Ca^{2+}$ mobilization response. In

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control cells transfected with pcDNA3 (mock), addition of ATP and UTP slightly induced $Ca^{2+}$ elevation, suggesting that low levels of endogenous P2Y receptors were present in HEK293T cells (**Fig. 5A**). Following the overexpression of $P2Y_2R$, 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 $P2Y_2R$-specific agonists (2SUTP, PSB1114 and MRS2768) these responses were even higher (**Fig. 5A**). In other controls, the $P2Y_1R$-specific agonist MRS2365 did not trigger $Ca^{2+}$ elevation in mock- or $P2Y_2R$-transfected cultures, and the $P2Y_2R$-specific agonists failed to elicit a response in $P2Y_1R$-transfected cultures (**Fig. 5A**). The dose-response curves of these agonists (MRS2365 for activating $P2Y_1R$, 2SUTP, PSB1114 and MRS3768 for activating $P2Y_2R$) were showed in **Supplementary Figure 1**. Based on these pharmacological studies, the $P2Y_2R$-specific agonists 2SUTP, PSB1114 and MRS2768 were employed for the specific activation of $P2Y_2R$ 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 $P2Y_2R$-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 $P2Y_2R$-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.

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Because $P2Y_2R$ 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 $P2Y_2R$ 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 $P2Y_2R$ 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 $G_q$-coupled signaling cascade. Activation of $P2Y_2R$ can stimulate PLC and increase $Ca^{2+}$ from intracellular stores (Van Kolen and Slegers, 2006), and the UTP-sensitive $P2Y_2R$ 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 $P2Y_2R$ in cortical neurons. Cortical neurons were pre-loaded 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 $P2Y_2R$ 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

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control for the stimulation of ERK1/2 phosphorylation. The above results suggested that the activation of $P2Y_2R$ can trigger $Ca^{2+}$ mobilization and induce ERK1/2 phosphorylation in cultured cortical neurons, which is consistent with previous findings with $P2Y_2R$ in muscle and other cell types.

Lastly, to ask if $Ca^{2+}$ and MAP kinase-dependent pathways participate downstream from $P2Y_2R$ 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 $P2Y_2R$ 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 $P2Y_2R$ 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 $P2Y_1R$ and $P2Y_2R$*

Our current results suggested a functional role of $P2Y_2R$ in cortical neurons by demonstrating its involvement in regulating cholinergic gene expression. Previously we found that cortical neurons also contain $P2Y_1R$ that can stimulate the expression of AChE and ChAT (Siow et al., 2010). This

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raised an interesting question about the significance of two P2Y receptors co-existing in cortical neurons. The co-existence of $P2Y_1R$ and $P2Y_2R$ in the same post-synaptic terminal were partially supported by our previous and current findings of double immuno-staining of PSD-95 with $P2Y_1R$ (Siow et al.,, 2010), and PSD-95 with $P2Y_2R$ (**Fig. 3**). Given their distinct ligand selectivity (ATP/ADP for $P2Y_1R$ and UTP for $P2Y_2R$) as well as their synaptic localization in neurons, we hypothesized a synergistic action between $P2Y_1R$ and $P2Y_2R$. To test this, cortical neurons were co-treated with the $P2Y_1R$-specific agonist MRS2365 and the $P2Y_2R$-specific agonists PSB1114 and MRS2768 and then analyzed for $Ca^{2+}$ mobilization and gene expression. A synergy in $P2Y_1R$ and $P2Y_2R$ actions was first demonstrated by a robust increase of intracellular $Ca^{2+}$ (**Fig. 7A**): the activation of $P2Y_1R$ alone (by MRS2365) or $P2Y_2R$ 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 $P2Y_1R$ activation, which was similar to that seen with the stimulation of $P2Y_2R$ (**Fig. 7B**). However, treatment with MRS2365 + PSB1114 (or MRS2365 + MRS2768) induced gene expression ~8-fold (**Fig. 7B**). The co-activation of $P2Y_1R$ and $P2Y_2R$ (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 $P2Y_1R$, and UTP for $P2Y_2R$) 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

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post-synaptic region in these neurons.

## Discussion

*Association of $P2Y_2R$ with synaptic regions and membrane rafts*

$P2Y_2R$ 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, $P2Y_2R$ 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 $P2Y_2R$ 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 $P2Y_2R$ 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 $P2Y_2R$ 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 $P2Y_1R$ and $P2Y_2R$ 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).

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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 $P2Y_2R$ may imply a post-synaptic signaling function in neurons, which might participate in the co-regulation of AChE and NR2A by $P2Y_1R$ (Siow et al., 2010). More importantly, the co-existence of $P2Y_1R$ and $P2Y_2R$ 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

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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 $P2Y_2R$ 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 $P2Y_1R$ and $P2Y_2R$, 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 $P2Y_1R$ is MRS2365 > 2-MeSADP > ADP > ATP, while that of $P2Y_2R$ is PSB1114 > MRS2768 > UTP ~ ATP.

*MAP kinase-dependent signaling pathway induced by $P2Y_2R$ activation*

The signaling pathway of $P2Y_2R$ is well-documented (Burnstock G, 2007). $P2Y_2R$ couples to PLC-β via $G\alpha_q$ to mediate $IP_3$ production, which results in the release of intracellular $Ca^{2+}$ and activation of PKC. Treatment of UTP ($P2Y_2R$ and $P2Y_4R$ agonist) and PSB1114 ($P2Y_2R$-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 $P2Y_1R$- or $P2Y_2R$-signaling in muscle cells and in $P2Y_1R$-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

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($P2Y_2R$-specific agonists) induced robust phosphorylation of ERK1 and ERK2 in a time-dependent manner. Our results suggest that MAP kinase signaling is activated by $Ca^{2+}$ 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 $Ca^{2+}$ mobilization and Erk phosphorylation in Fig. 6 were higher than that of $P2Y_2R$-specific agonist PSB1114. This might be attributed to the simultaneously activation of $P2Y_4R$ by UTP in the cultures. From our preliminary results, $P2Y_4R$ was also existed in mature cortical neurons. Since activation of $P2Y_4R$ could also trigger $IP_3$ formation and $Ca^{2+}$ mobilization via $G_{q/11}$ coupling (Unpublished data). This is a critical reason for using the $P2Y_2R$-specific agonists for testing rather than UTP in the current study.

Activation of $P2Y_2R$ 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 $P2Y_1R$ via Raf/MEK/ERK pathway in muscles (Choi et al., 2003) and in cortical neurons (Siow et al., 2010). For $P2Y_2R$, 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 $P2Y_2R$ 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

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(**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 $P2Y_1R$ and $P2Y_2R$ 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 $P2Y_2R$ 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, $P2Y_2R$ activation induced *ACHE* gene expression in muscle cells (Tung et al., 2004), but the AChE $G_4$ isoform and AChE enzymatic activities were not changed upon treatment with UTP and other $P2Y_2R$ specific agonists. This suggests that ATP-, UTP- and $P2Y_2R$-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 $P2Y_2R$ activation. Our data further showed that the treatment of cultured cortical neurons with UTP and $P2Y_2R$-specific agonists tended to increase the protein level of AChE slightly more than that of PRiMA, raising the

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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, $P2Y_2R$ was shown to be expressed together with ATP-sensitive $P2Y_1R$ in cultured cortical neurons. Activation of $P2Y_2R$ 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 $P2Y_1R$ and $P2Y_2R$ in cortical neurons were found to be synergistic, and our results thus provide evidence for the co-existence of (at least) $P2Y_1R$ and $P2Y_2R$ 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 $P2Y_2R$ 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 $P2Y_1R$ (~318 bp), $P2Y_2R$ (~200 bp), $P2Y_4R$ (~550 bp) and $P2Y_6R$ (~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 $P2Y_2R$-encoding cDNA or empty vector were triple stained by TO-PRO-3 (for cell nuclei), anti-$P2Y_2R$ and/or anti-Xpress antibodies (see *Methods*) and examined by confocal microscopy. In blocking experiments, the antigenic peptide from $P2Y_2R$ was pre-incubated with anti-$P2Y_2R$ before immuno-staining. Representative images shown; n = 4, scale bar = 10 μm. [D]: Western blotting confirmed that anti-$P2Y_2R$ stained an ~65 kDa protein band corresponding to $P2Y_2R$ 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 $P2Y_2R$ 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 $P2Y_2R$ in rat cortical neurons. [A]: To demonstrate the

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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 $P2Y_2R$ 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 $P2Y_2R$ during the differentiation of cultured cortical neurons (5-25 DIV) was determined by Western blotting. The level of $P2Y_2R$ 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 $P2Y_2R$ in cultured cortical neurons. Three-week-old cultured cortical neurons were fixed and double-stained with anti-$P2Y_2R$ (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 $P2Y_2R$ and PSD-95 (yellow). Representative images shown; n = 6, scale bars = 20 μm.

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**FIGURE 4.** Association of $P2Y_2R$ 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 $P2Y_1R$ or $P2Y_2R$ with membrane rafts (fractions 5–7). Flotillin-2 was used a raft-marker, while $AChE_T$, 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 $P2Y_1R$ and $P2Y_2R$ 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 $P2Y_2R$ in cortical neurons. [A]: To demonstrate agonist specificity, HEK293T cells were transfected with pcDNA3 (mock control), $P2Y_1R$ or $P2Y_2R$ cDNAs, and then labeled with Fluo 4-AM to monitor $Ca^{2+}$ mobilization. The general agonists used were ATP (100 nM) and UTP (100 nM) and the receptor-specific agonists were MRS2365 (100 nM) for $P2Y_1R$, and 2SUTP (100 nM), PSB1114 (100 nM) and MRS2768 (100 nM) for $P2Y_2R$. [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

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show, antibody staining revealed the expression of $AChE_T$ (~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 $P2Y_2R$-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 $AChE_T$, ChAT and

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PRiMA mRNAs. Data are normalized and expressed as ratios relative to the basal reading (DMSO/$H_2O$-treated control set as 1); mean ± SEM, n = 4.

**FIGURE 7.** Synergistic effect of $P2Y_1R$ and $P2Y_2R$ in activating cholinergic gene expression. [A]: Cortical neurons at 14 DIV were labeled with Fluo 4-AM and then treated with $P2Y_1R$ agonist alone (10 μM MRS2365), $P2Y_2R$ agonist alone (10 μM PSB1114), or the two receptor agonists combined (PSB1114 + MRS2365) before $Ca^{2+}$ 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 $P2Y_2R$-specific agonist) or MRS2768 + MRS2365, for 15 hours. Total mRNAs were extracted and the expression of cholinergic genes $AChE_T$, ChAT and PRiMA 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 $P2Y_1R$ and $P2Y_2R$ led to higher expression of $AChE_T$, ChAT and PRiMA proteins than the stimulation of just one of the two receptors. Data are normalized and expressed relative to the basal reading with $H_2O$-treated control (set as 1); mean ± SEM, n = 4.

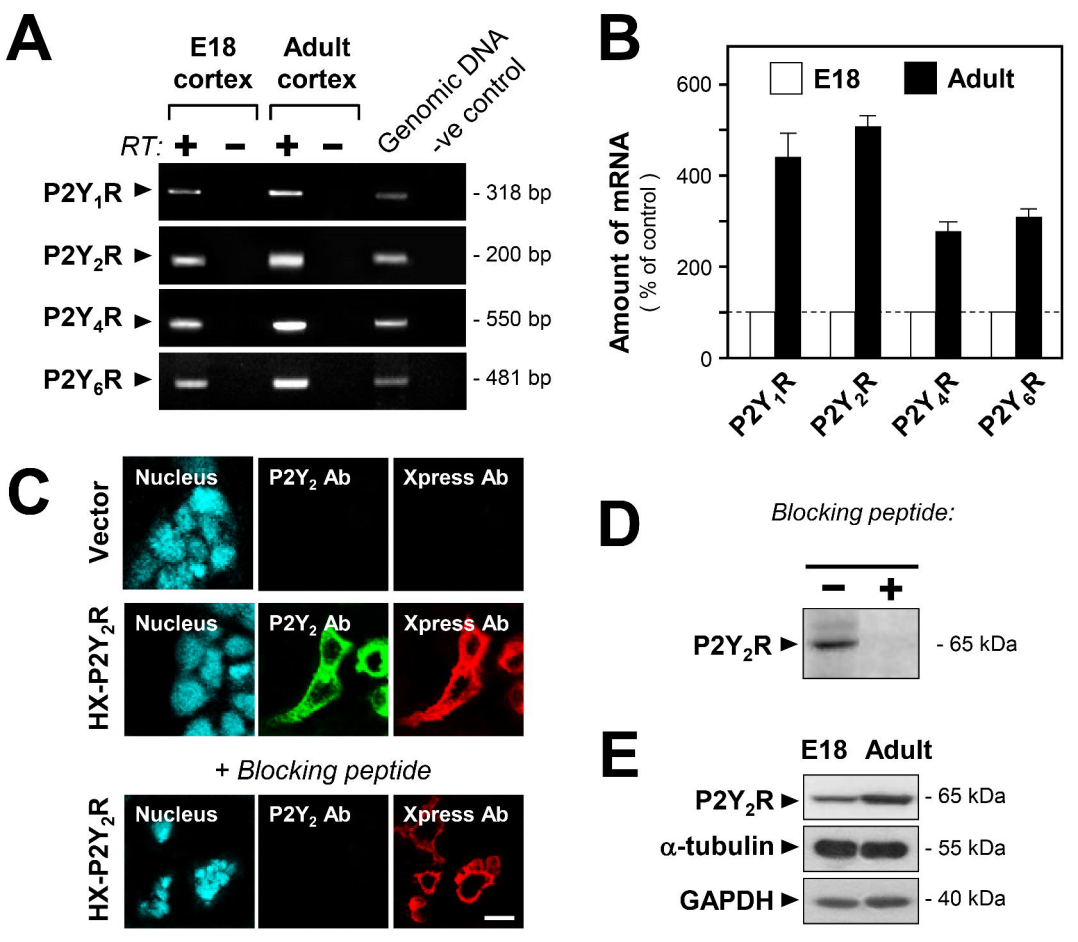
A
E18 cortex
Adult cortex
Genomic DNA
-ve control
RT: + − + −
P2Y1R
P2Y2R
P2Y4R
P2Y6R
- 318 bp
- 200 bp
- 550 bp
- 481 bp
B
E18
Adult
Amount of mRNA
( % of control )
600
400
200
0
P2Y1R
P2Y2R
P2Y4R
P2Y6R
C
Vector
HX-P2Y2R
Nucleus
P2Y2 Ab
Xpress Ab
+ Blocking peptide
D
Blocking peptide:
− +
P2Y2R
- 65 kDa
E
E18 Adult
P2Y2R
α-tubulin
GAPDH
- 65 kDa
- 55 kDa
- 40 kDa

Figure 1

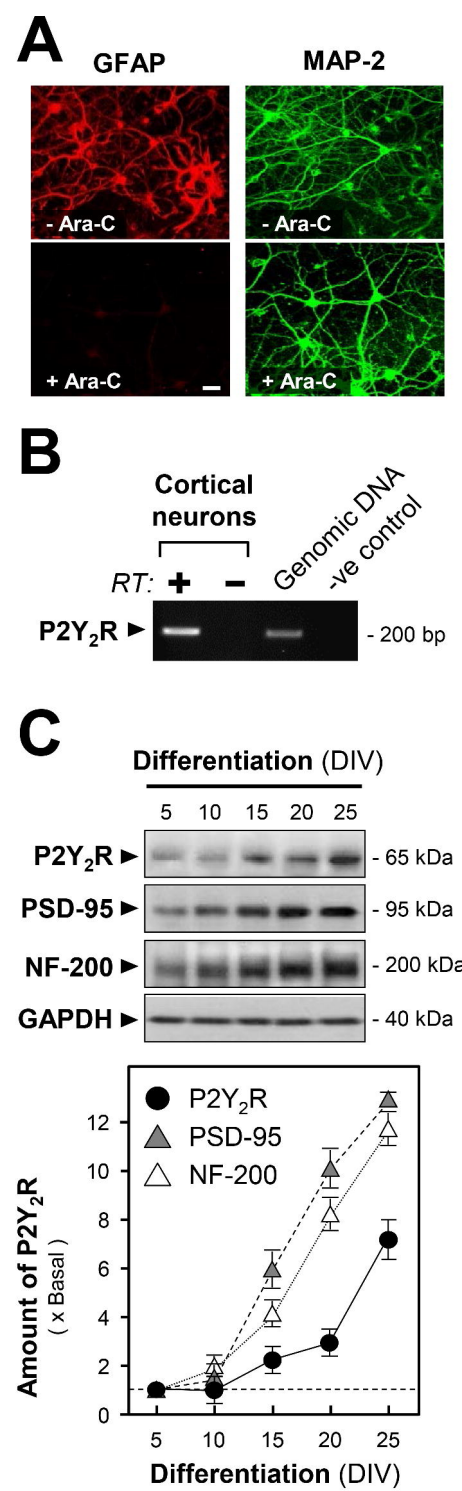
A
GFAP
MAP-2
- Ara-C
- Ara-C
+ Ara-C
+ Ara-C
B
Cortical neurons
Genomic DNA -ve control
RT: + −
P2Y2R ►
- 200 bp
C
Differentiation (DIV)
5 10 15 20 25
P2Y2R ►
- 65 kDa
PSD-95 ►
- 95 kDa
NF-200 ►
- 200 kDa
GAPDH ►
- 40 kDa
P2Y2R
PSD-95
NF-200
Amount of P2Y2R
( x Basal )
0 2 4 6 8 10 12
5 10 15 20 25
Differentiation (DIV)

*Figure 2*

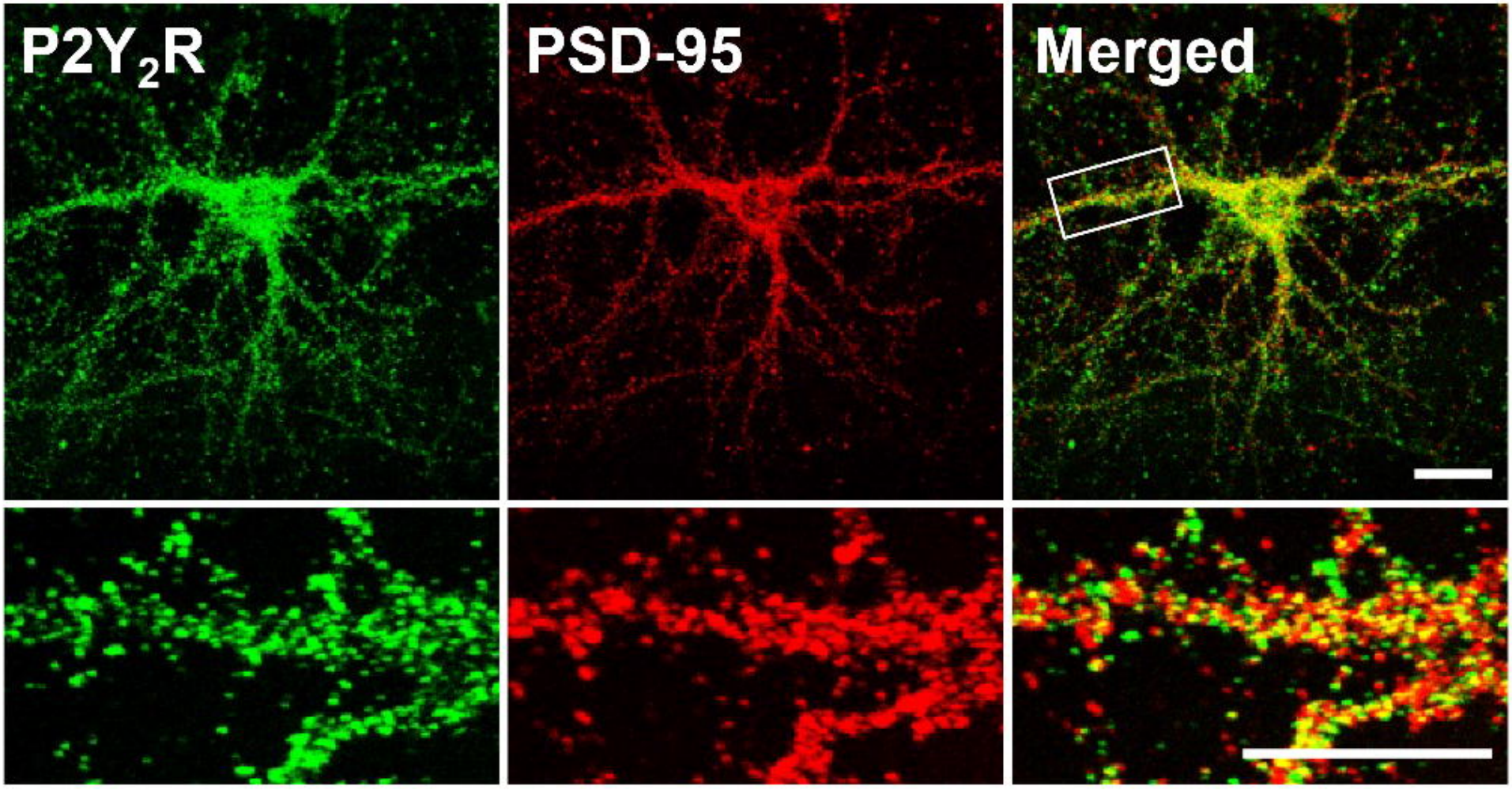


Figure 3

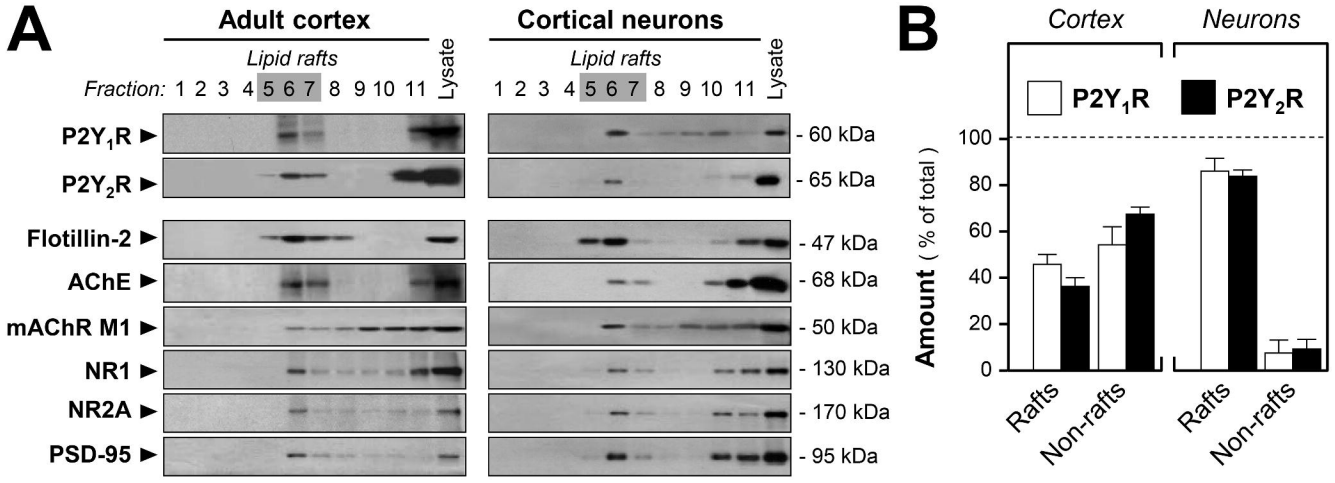
A
Adult cortex
Cortical neurons
Lipid rafts
Lipid rafts
Fraction: 1 2 3 4 5 6 7 8 9 10 11 Lysate
1 2 3 4 5 6 7 8 9 10 11 Lysate
P2Y1R ►
P2Y2R ►
Flotillin-2 ►
AChE ►
mAChR M1 ►
NR1 ►
NR2A ►
PSD-95 ►
- 60 kDa
- 65 kDa
- 47 kDa
- 68 kDa
- 50 kDa
- 130 kDa
- 170 kDa
- 95 kDa
B
Cortex
Neurons
P2Y1R
P2Y2R
Amount ( % of total )
100
80
60
40
20
0
Rafts
Non-rafts
Rafts
Non-rafts

Figure 4

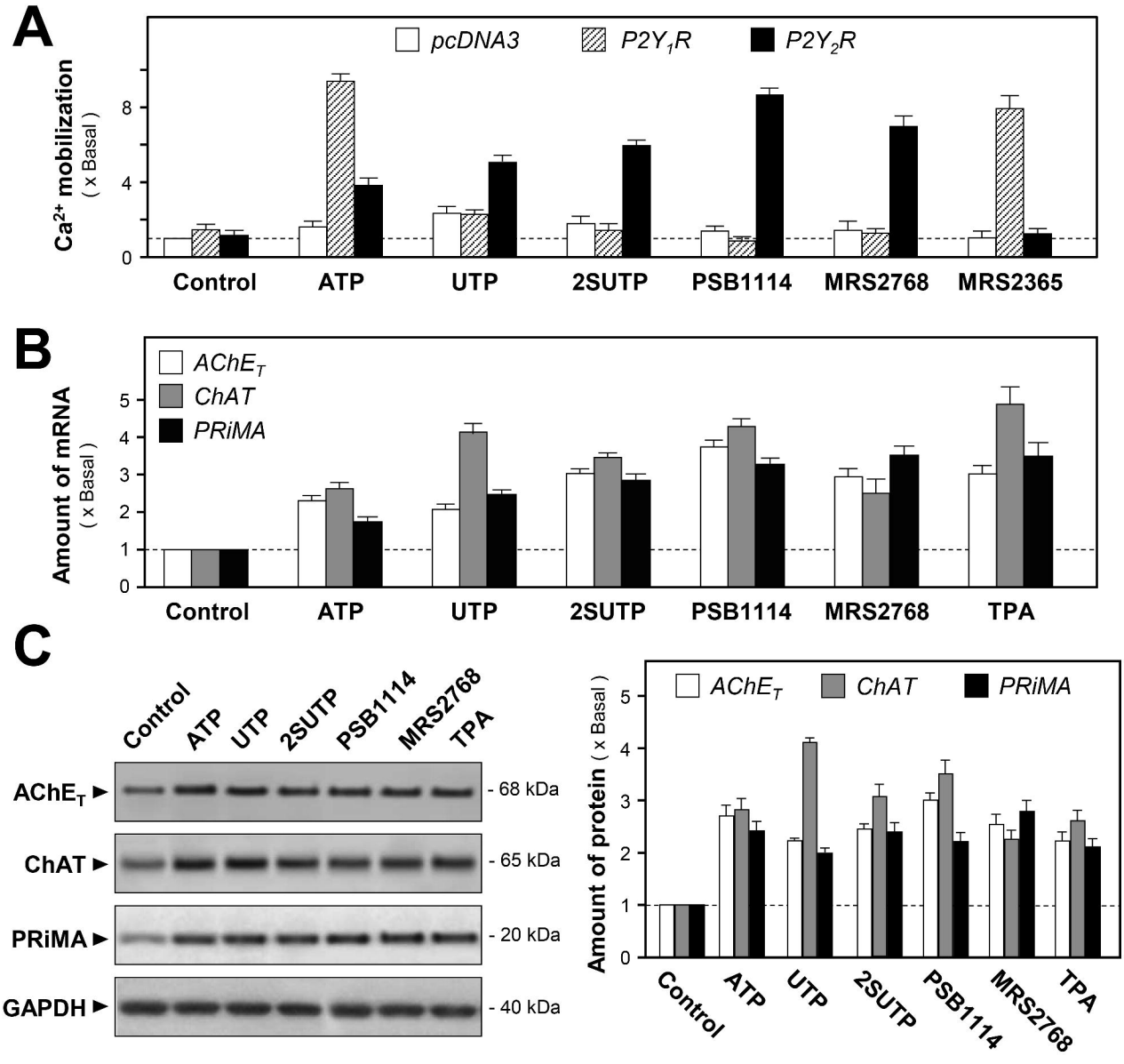
A
pcDNA3
P2Y1R
P2Y2R
Ca2+ mobilization
( x Basal )
0
4
8
Control
ATP
UTP
2SUTP
PSB1114
MRS2768
MRS2365
B
AChET
ChAT
PRiMA
Amount of mRNA
( x Basal )
0
1
2
3
4
5
Control
ATP
UTP
2SUTP
PSB1114
MRS2768
TPA
C
Control
ATP
UTP
2SUTP
PSB1114
MRS2768
TPA
AChET ►
- 68 kDa
ChAT ►
- 65 kDa
PRiMA ►
- 20 kDa
GAPDH ►
- 40 kDa
AChET
ChAT
PRiMA
Amount of protein ( x Basal )
0
1
2
3
4
5
Control
ATP
UTP
2SUTP
PSB1114
MRS2768
TPA

*Figure 5*

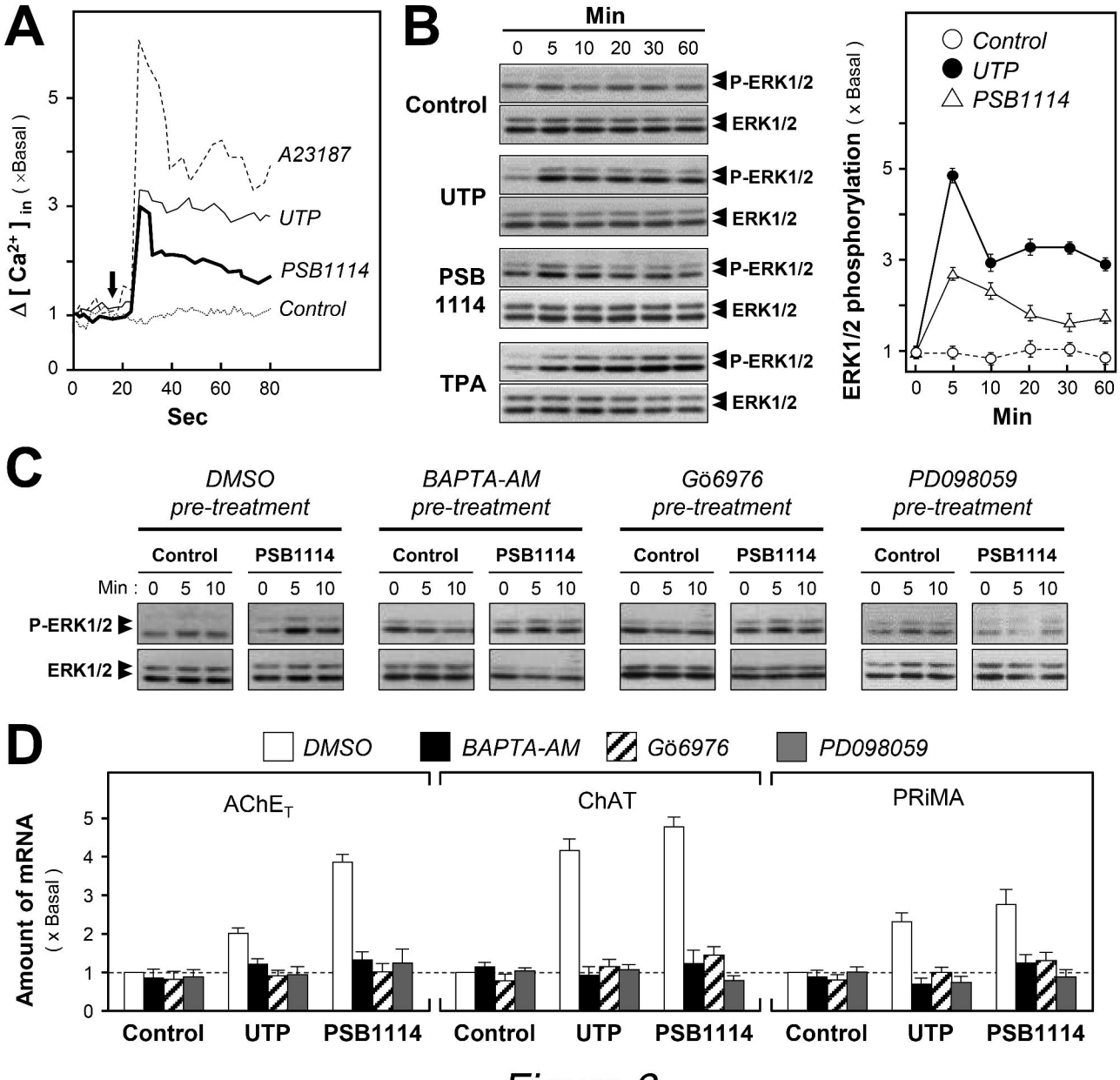
A
Δ [ Ca2+ ] in ( ×Basal )
A23187
UTP
PSB1114
Control
Sec
B
Min
0 5 10 20 30 60
Control
UTP
PSB 1114
TPA
P-ERK1/2
ERK1/2
Control
UTP
PSB1114
ERK1/2 phosphorylation ( x Basal )
Min
C
DMSO pre-treatment
BAPTA-AM pre-treatment
Gö6976 pre-treatment
PD098059 pre-treatment
Control
PSB1114
Min : 0 5 10
P-ERK1/2
ERK1/2
D
DMSO
BAPTA-AM
Gö6976
PD098059
AChET
ChAT
PRiMA
Amount of mRNA ( x Basal )
Control
UTP
PSB1114

Figure 6

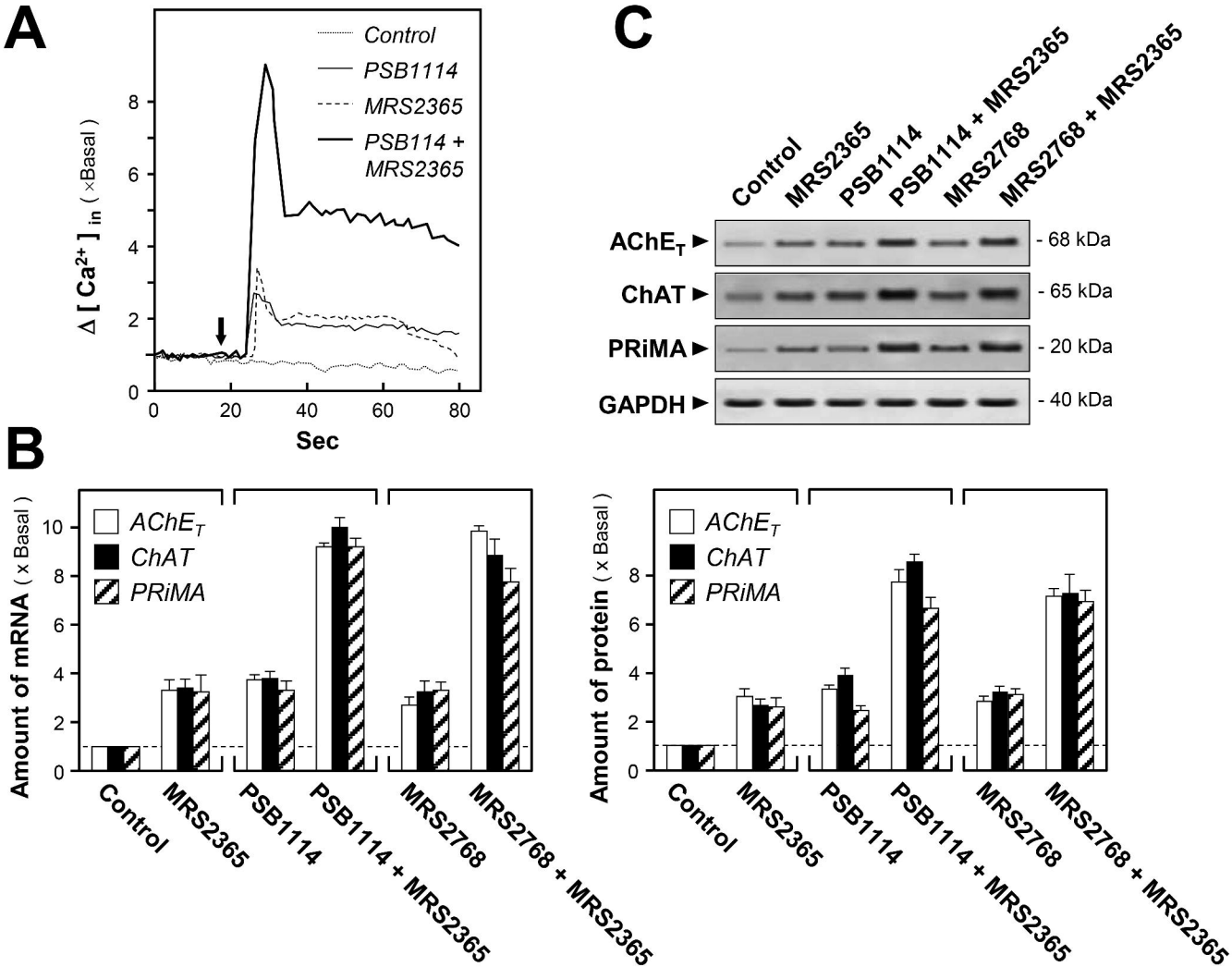
A
Control
PSB1114
MRS2365
PSB114 + MRS2365
Δ [ Ca2+ ] in ( ×Basal )
Sec
C
Control
MRS2365
PSB1114
PSB1114 + MRS2365
MRS2768
MRS2768 + MRS2365
AChET ►
ChAT ►
PRiMA ►
GAPDH ►
- 68 kDa
- 65 kDa
- 20 kDa
- 40 kDa
B
Amount of mRNA ( x Basal )
AChET
ChAT
PRiMA
Control
MRS2365
PSB1114
PSB1114 + MRS2365
MRS2768
MRS2768 + MRS2365
Amount of protein ( x Basal )

Figure 7