Immunogold electron microscopic evidence of in situ formation of homo- and heteromeric purinergic adenosine A\(_1\) and P2Y\(_2\) receptors in rat brain

Kazunori Namba\(^1\)\(^2\)*, Tokiko Suzuki\(^1\)\(^3\), Hiroyasu Nakata\(^1\)

**Abstract**

**Background:** Purines such as adenosine and ATP are now generally recognized as the regulators of many physiological functions, such as neurotransmission, pain, cardiac function, and immune responses. Purines exert their functions via purinergic receptors, which are divided into adenosine and P2 receptors. Recently, we demonstrated that the G\(_{i/o}\)-coupled adenosine A\(_1\) receptor (A\(_1\)R) and G\(_{q/11}\)-coupled P2Y\(_2\) receptor (P2Y\(_2\)R) form a heteromeric complex with unique pharmacology in co-transfected human embryonic kidney cells (HEK293T). However, the heteromeric interaction of A\(_1\)R and P2Y\(_2\)R in situ in brain is still largely unknown.

**Findings:** In the present study, we visualized the surface expression and co-localization of A\(_1\)R and P2Y\(_2\)R in both transfected HEK293T cells and in rat brain by confocal microscopy and more precisely by immunogold electron microscopy. Immunogold electron microscopy showed the evidence for the existence of homo- and hetero-dimers among A\(_1\)R and P2Y\(_2\)R at the neurons in cortex, cerebellum, and particularly cerebellar Purkinje cells, also supported by co-immunoprecipitation study.

**Conclusion:** The results suggest that evidence for the existence of homo- and hetero-dimers of A\(_1\)R and P2Y\(_2\)R, not only in co-transfected cultured cells, but also in situ on the surface of neurons in various brain regions. While the homo-dimerization ratios displayed similar patterns in all three regions, the rates of hetero-dimerization were prominent in hippocampal pyramidal cells among the three regions.

**Background**

The adenosine A\(_1\) receptor (A\(_1\)R) is known to regulate Ca\(^{2+}/K^+\) channels, adenylyl cyclase, and phospholipase C by coupling to G\(_{i/o}\) proteins [1]. In hippocampal astrocytes, P2Y\(_1\)R- and P2Y\(_2\)R-mediated Ca\(^{2+}\) responses differentially show two forms of activity-dependent negative feedback of synaptic transmission via the phospholipase C beta-IP\(_3\) pathway [2]. Today, the homo- or hetero-dimers of many kinds of GPCRs have been reported [3]. We previously demonstrated that A\(_1\)R associates with P2Y\(_1\)R in co-transfected HEK293T cells and in rat brain homogenates, whereby a P2Y\(_1\)R agonist stimulates A\(_1\)R signaling via G\(_{i/o}\) [4,5]. Furthermore, in HEK293T cells co-transfected with A\(_4\)R and P2Y\(_3\)R, the heterodimers display synergistic increases in Ca\(^{2+}\) signaling, whereby simultaneous activation of the two receptors attenuates A\(_1\)R signaling via G\(_{i/o}\) but synergistically enhances P2Y\(_3\)R signaling via G\(_{q/11}\) [6]. Also, the simultaneous activation of endogenous A\(_1\)R and P2Y\(_2\)R in DDT1MF-2 cells synergistically increases translocation of protein kinase C [7]. Because A\(_1\)R are widely expressed in brain [5], it is likely that these receptors also associate directly in situ; however, direct evidence of their dimerization or precise co-localization in brain has yet to be demonstrated. The aim of the present study is to determine whether A\(_1\)R and P2Y\(_2\)R associate with each other in rat brain by co-immunoprecipitation and looking for receptor complexes via immunogold electron microscopy (IEM).

---

* Correspondence: nambakazunori@kankakuki.go.jp
1 Department of Molecular Cell Signaling, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183-8526, Japan
Full list of author information is available at the end of the article

© 2010 Namba et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Methods

Double immunostaining of A1R/P2Y2R in HEK293T cells and rat brain sections

Double immunostaining using anti-HA 3F10 mAb rat antibody (anti-HA) and anti-Myc 9E10 mAb mouse antibody (anti-Myc) in HA-A1R and Myc-P2Y2R co-transfected HEK293T cells were performed as previously described [6]. Cells were washed and then stained with Alexa 568-conjugated goat anti-rat IgG antibody (1:200, Invitrogen, Carlsbad, CA) for A1R or Alexa 488-conjugated goat anti-mouse IgG antibody (1:200, Invitrogen) for P2Y2R. The characterization of antibodies for rat brain sections was previously reported, although the rabbit polyclonal anti-P2Y2R antibody (anti-P2Y2R; 1 μg/ml, Alomone Labs, Jerusalem, Israel) was used instead of the rabbit polyclonal anti-P2Y1R antibody [5,8].

Immunoprecipitation and western blotting of rat brain homogenates

Eight-week-old male Wistar rats were decapitated under anesthesia (Nembutal; 30 mg/kg i.v.), and cortical, hippocampal, and cerebellar tissues were dissected out. The tissues were homogenized with a Polytron homogenizer in 50 mM Tris-acetate, pH 7.4, containing a protease inhibitor cocktail (Roche Applied Science, Manheim, Germany), and the resulting cell suspensions were centrifuged at 30,000 × g for 30 min at 4°C. The pellets were solubilized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 300 mM NaCl and a protease inhibitor cocktail) for 60 min at 4°C. The mixture was centrifuged at 18,500 × g for 20 min at 4°C, and the supernatant pre-cleared with Protein G-Sepharose™4 Fast Flow (Amersham Bioscience, Piscataway, NJ). The lysate was incubated with rabbit polyclonal anti-A1R antibody (anti-A1R; 1 μg/ml, Sigma-Aldrich, St. Louis, MO) for 60 min at 4°C. Protein G-Sepharose was added to the mixture, and the incubation continued for an additional 120 min. Protein G-Sepharose was recovered by centrifugation and washed three times with lysis buffer. Immunoprecipitates were eluted with SDS-PAGE sample buffer, resolved by 12% SDS-PAGE, and electrotransferred to nitrocellulose membranes. Receptors on the blot were detected using anti-A1R and anti-Myc for 3 h at 4°C. After washing with PBS, cells were incubated with 10-nm gold particle-conjugated goat anti-rat IgG antibody (rat IgG-10, 1:1000, BBI International, Lakewood, CO) and 5-nm gold particle-conjugated goat anti-mouse IgG antibody (mouse IgG-5, 1:1000, BBI International) for 4 h at 4°C. After washing, the cells were fixed with 2.5% glutaraldehyde in 0.15 M sodium cacodylate, pH 7.4 for 2 h, washed, and post-fixed with 1% osmium tetroxide for 4 h at room temperature. The cells were then dehydrated and embedding resin (Epon 812; NISsIN EM, Tokyo, Japan). Specimens were observed with an H7500 electron microscope (Hitachi, Japan). We quantified the gold staining as follows: The gene-transfected HEK293T cells with the highest numbers of total immuno-reacted gold particles were defined as 100% labeling. Because the co-transfected HEK293T cells that displayed unique pharmacology in our previous study [6] exhibited more than 20% hetero-dimeric gold particles, we used this number as a threshold in the current study. Thus, cells with more than 20% hetero-dimeric particles were defined as being “significantly stained”, and those with 20% or less were defined as “not significantly stained”.

Post-embedding immunogold electron microscopy of brain tissues

Dissected brain tissues were cut into 1.0 mm³ blocks that were then incubated with lead (II) acetate (Sigma-Aldrich) buffer for 1 h at room temperature, dehydrated through a series of graded ethanol, and embedded in LR-white (NISsIN EM). Ultra thin sections (40 nm) were mounted on 200-mesh nickel grids (NISsIN EM) and incubated in PBS containing 1% BSA for 10 min. After immunostaining with primary antibodies, each specimen was incubated with mouse IgG-5- and IgG-10-nm gold particle-conjugated goat anti-rabbit IgG antibody (rabbit IgG-10) for 6 h at 4°C. For controls, transfected HEK293T cells were embedded with LR-white under the same conditions as described above. After incubation at 4°C for 12 h with anti-HA (10 μg/ml) and anti-Myc (10 μg/ml), samples were washed with 1% BSA/PBS. After incubation with gold particle-conjugated secondary antibodies for 6 h at 4°C, sections were stained with uranyl acetate for 10 min. “Significant heteromeric staining” was defined as more than 20% of the total number of immuno-reacted gold particles at the cell surface occurring in heteromeric clusters.

Comparison of the numbers of monomers, homo-dimers, and hetero-dimers

The numbers of immunogold particles at the cell surface of each cell type were determined. We defined single particles located independently as monomers (A1R and P2Y2R in Figure 1), complexes composed of clusters
of the same-sized gold particles as “homo-dimers” (A₁R-A₁R or P₂Y₂R-P₂Y₂R in Figure 1), and those of different sized gold particles as “hetero-dimers” (A₁R-P₂Y₂R in Figure 1). Separate calculations were made of particles in cortical neurons (Figure 1A), hippocampal pyramidal neurons (Figure 1B), and Purkinje cells (Figure 1C); gold particles were counted in three cells in each region. We also counted immunogold particles in co-transfected HEK293T cells (please see above, and Figure 1D). The total number of immunoreactive gold particles on each cell surface was defined as 100%. Each column represents the average frequency (± SD) from three cells. Raw data are shown in the tables under the graphs. Data are means of three independent experiments.

Figure 1 Bar graphs comparing the relative distributions of A₁R(A₁)- and P₂Y₂R(Y₂)-immunoreactive elements in each brain region (A-C) and in transfected HEK293T cells (D). The P₂Y₂R-P₂Y₂R, A₁R-A₁R and A₁R-P₂Y₂R dimers are indicated by Y₂-Y₂, A₁-A₁ and A₁-Y₂, respectively. Total number of immunoreactive gold particles on the cell surface was defined as 100%. Each column represents the average frequency (± SD) from three cells. Raw data are shown in the tables under the graphs. Data are means of three independent experiments.
the three photos of each specimen containing whole cells were selected randomly for comparison.

**Results**

**Co-localization of A1R and P2Y2R in transfected HEK293T cells**

The co-localization of A1R and P2Y2R in co-transfected HEK293T cells was examined by double immunostaining of HA-A1R and Myc-P2Y2R as a comparison experiment for the localization of these receptors in brain tissues (Figure 2). Both receptors were localized mainly on cell surface and cytosolic membranes, but not in the nucleus (Figure 2A, B). Merged images showed their co-localization mainly in cell membranes (Figure 2C). No signals were observed in non-transfected HEK293T cells, indicating that the immunoreactivity observed in Figure 2 was specific to the transfected HEK293T cells.

**Figure 2** Co-localization of A1R and P2Y2R

A-C. Confocal images of double immunostained Myc-P2Y2R (A; green), HA-A1R (B; red), and their merge (C; yellow) in co-transfected HEK293T cells. The co-localization of HA-A1R and Myc-P2Y2R is evident at the cell surface membrane (small arrow). D-L. Confocal images of double immunofluorescence staining in several rat brain regions. P2Y2R (D, G, J; red) and A1R (E, H, K; green) immunoreactivities were detected in Purkinje cells (D-F), cerebellar nuclei (G-I), and hippocampal CA3 pyramidal cells (J-L). Co-localizations of A1R and P2Y2R (F, I, L; yellow) were detected in the soma (large arrows) of all tissues, in dendrites of the Purkinje cells, and in neurons of the cerebellar nuclei (arrowheads). Yellow bar indicates 500 μm (A-C) and white bar indicates 100 μm (D-L). Mol: cerebellar molecular layer, Gr: cerebellar granule cell layer. Fluorescent images were collected via confocal laser scanning microscopy (Zeiss LSM410, Carl Zeiss, Oberkochen, Germany) each 10-μm optical slice consisted of a stack of 20 0.5-μm thick sections. Serial optical sections were recorded using an air objective lens of 40x, numerical aperture; 0.6.
was specific to the expressed receptors (data not shown). These results suggest that both receptors were expressed on cell membranes.

**Immunohistochemical studies in rat brain**

We examined the expression of A1R and P2Y2R in brain using immunohistochemical analyses (Figure 2). The specificity of the antibodies against A1R and P2Y2R was confirmed by the immunocytochemistry of recombinant receptor-expressing cell lines, i.e. antibodies used in this study showed no cross-labeling in A1R- and P2Y2R-transfected HEK293T cells (data not shown). Prominent staining of A1R and P2Y2R were observed especially in Purkinje cells (Figure 2D-F), interposed cerebellar nuclei (Figure 2G-H), and hippocampal pyramidal cells (Figure 2J-L). Comparatively high immunoreactivities were also detected in the piriform cortex, amygdala, hypothalamus, and brainstem (data not shown). Their expressions were mainly restricted to cell bodies and neuronal dendrites. Importantly, co-localization of A1R and P2Y2R in the cerebellum was observed in cell bodies, except in the nuclear region, in the Purkinje cells and those of the interposed cerebellar lobule nucleus (Figure 2D-I). In the hippocampal region, pyramidal cell bodies, especially the cell surface membranes, in CA1, CA2, CA3, and the dentate gyrus (CA3; Figure 2J-L, others; data not shown) were intensely stained for both A1R and P2Y2R. Similar staining patterns were seen in cell bodies of neurons in the cerebral cortex (data not shown).

**Co-immunoprecipitation of A1R and P2Y2R from rat brain**

Next, we examined whether A1R and P2Y2R are associated with one another in several brain regions using immunoprecipitation with anti-A1R followed by immunoblotting with both A1R and P2Y2R antibodies (Figure 3). A1R and P2Y2R immunoreactivities were present in all three rat brain regions examined (Figure 3A, B, F). Moreover, in these same regions, anti-A1R were capable of co-precipitating P2Y2R (Figure 3D), indicating that A1R and P2Y2R are associated with one another in rat cortex, cerebellum, and hippocampus. The absence of these immunoreactive bands in the presence of P2Y2R antigen peptides (Figure 3C, E) is evidence of their specificity of the antibodies. The specificity of the anti-A1R was confirmed by immunocytochemistry of mock-transfected HEK293T cells, and no specific band was detected (data not shown).

**Immunogold electron microscopic observations of HA-A1R and Myc-P2Y2R expressed in HEK293T cells**

The immunogold particles were localized singly or in clusters, indicating that both HA-A1R and Myc-P2Y2R form monomers and homo-dimers. Specificities of the gold-labeled anti-HA and anti-Myc were demonstrated by incubating A1R-transfected HEK293T cells with a mixture of both antibodies, and showed that only A1R-labeled particles were present (Figure 4D). No significant immunoreactivity was detected with both anti-HA and anti-Myc in mock-transfected HEK293T cells or with only secondary antibodies (no primary antibodies) in HA-A1R-transfected HEK293T cells (data not shown). Also, when Myc-P2Y2R-transfected HEK293T cells were
Figure 4 Immunogold electron microscopy of A1R and P2Y2R visualized using nanogold particles in transfected HEK293T cells (A-D) and rat brain (E-G). A: Localization of HA-A1R (large particles) detected with anti-HA in HA-A1R-transfected HEK293T cells. B: Localization of Myc-P2Y2R (small particles) detected with anti-Myc in Myc-P2Y2R-transfected HEK293T cells. C: Anti-HA and anti-Myc immuno-localization of HA-A1R and Myc-P2Y2R in co-transfected HEK293T cells. D: HA-A1R-transfected HEK293T cells incubated with both anti-HA and anti-Myc. E-G: Localization of A1R and P2Y2R in cortical pyramidal cells (E), Purkinje cells (F), and hippocampal pyramidal cells (G) detected with both anti-A1R and anti-P2Y2R. Arrows indicate two adjacent receptors on the cell membrane. Bars represent 100 nm. CM, cell membrane; CP, cytoplasm.
incubated with both anti-HA and anti-Myc, single particles (monomers) were scattered all over the cells, whereas co-localized, equal-sized particles of Myc-P2Y2R (homo-dimers) were only occasionally seen (data not shown). In HEK293T cells co-transfected with both HA-A1R and Myc-P2Y2R, clusters of different-sized particles were observed mainly at the cell surface (Figure 4C) might be suggestive that they form heteromeric complexes.

**Immunogold electron microscopic observations of A1R and P2Y2R expressed in rat brain**

We incubated post-embedded, primary antibody-stained rat brain tissues with secondary antibodies labeled with mouse IgG-5 for A1R and rabbit IgG-10 for P2Y2R. As negative controls, tissues were stained with only secondary antibodies conjugated with different sized gold particles; no significant immunoreactivities were observed under the experimental conditions used in this study (data not shown). As in the transfected HEK293T cells, we observed clusters of different-sized gold particles at cytoplasmic membranes in cell bodies, indicating the presence of heteromeric complexes of endogenous A1R and P2Y2R in rat brain (Figure 4E-G). Significant immunoreactivity was detected in Purkinje cells (Figure 4F) and hippocampal pyramidal cells (Figure 4G). Hetero- and homo-dimers were detected in significant numbers at the cell surface in both transfected HEK293T cells and native brains.

**Comparison of the frequencies of monomers, homo-dimers, and hetero-dimers**

We counted gold particles on the surfaces of cells in the cortex, cerebellum, and hippocampus and classified them as monomers (A1R or P2Y2R), homo-dimers (A1R-A1R or P2Y2R-P2Y2R), or hetero-dimers (A1R-P2Y2R). While the homo-dimerization ratios (A1R-A1R/P2Y2R-P2Y2R) displayed similar patterns in all three regions (Figure 1A-C), the rates of hetero-dimerization were prominent in hippocampal pyramidal cells among the three regions.

**Discussion**

The present study provides the first detailed evidence of an interaction between endogenous A1R and P2Y2R in brains using co-immunoprecipitation and IEM. The homo-dimerization of A1R was previously analyzed in our laboratory by computational prediction, co-immunoprecipitation, and BRET analysis [9]. In the present study, we might suggest the existence of homodimers (A1R-A1R and P2Y2R-P2Y2R) using IEM. Very interestingly, the percentage of A1R homodimers was higher than that of P2Y2R in both rat brain and transfected HEK293T cells (Figure 1). By contrast, the ratios of heteromeric gold-particle clusters were different in the cortex, hippocampus, and cerebellum. Importantly, both homo-dimeric and hetero-dimeric gold-particles were much fewer at inner cytoplasmic membranes than at the cell surface (data not shown). In general, most GPCRs dimers have been observed on the cell surface [10,11]. Total numbers of hetero-dimers observed on the cell surface and in the cytoplasm were obviously different (data not shown) and may reflect the process of receptor maturation and association of the A1R-P2Y2R complex.

In the hippocampal region, the strong presence of hetero-dimers coincided with the relative signal intensity of the co-immunoprecipitation band (Figure 3D lane 3). In the previously reported electron microscopic analysis of A1R and P2Y1R co-localization in hippocampus, the A1R density was relatively higher than that of P2Y1R at the presynaptic membrane [12]. They suggested that the hetero-dimerization or cross-talk of A1R and P2Y1R is involved in regulation of glutamate release. The relative distributions of immunoreactivities for GABA_B2 and GABA_B1 were also different in the basal ganglia and globus pallidus/substantia nigra, which suggests the possible co-existence and hetero-dimerization of two types of receptors at various pre-/postsynaptic sites [13]. From the present study, it can be speculated that the A1R/P2Y1R hetero-oligomer might be responsible for down regulation, via hippocampal Ca^{2+} secretion, of synaptic functions [14]. The abundant formation of A1R/P2Y2R hetero-oligomers in hippocampus revealed in this study supports the idea that the unique signal transduction generated by hetero-dimerization, including the enhancement of Ca^{2+} signaling via G11', observed in transfected cells also occurs in hippocampus.

**List of abbreviations**

GPCR: G protein-coupled Receptor; A1R: A1 adenosine receptor; P2Y1R: P2Y1 purinergic receptor; P2Y2R: P2Y2 purinergic receptor; IEM: immunogold electron microscopy

**Acknowledgements**

We thank Masumi Ichikawa and Kyoko Ajiki at the Tokyo Metropolitan Institute for Neuroscience for their help with the electron microscopy. This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant number: 16300125, 00041830, 00041830

**Authors' contributions**

KN carried out all experiments, prepared the figures and drafted the manuscript. TS assisted immunostaining experiment and in manuscript
revising. NH was responsible for experimental design and revised and polished the manuscript. All authors have read and approved final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 27 July 2010  Accepted: 29 November 2010
Published: 29 November 2010

References
1. Ralevic V, Burnstock G: Receptors for purines and pyrimidines. Pharmacol Rev 1998, 50(3):413-492.
2. Fam SR, Gallagher CJ, Kalia LV, Salter MW: Differential frequency dependence of P2Y_{1} and P2Y_{2} mediated Ca^{2+} signaling in astrocytes. J Neurosci 2003, 23(11):4437-4444.
3. Bouvier M: Oligomerization of G-protein-coupled transmitter receptors. Nat Rev Neurosci 2001, 2(4):274-286.
4. Yoshikawa K, Saitoh Q, Nakata H: Heteromeric association creates a P2Y-like adenosine receptor. Proc Natl Acad Sci USA 2001, 98(13):7617-7622.
5. Yoshikawa K, Hosoda R, Kuroda Y, Nakata H: Hetero-oligomerization of adenosine A_{1} receptors with P2Y_{2} receptors in rat brains. FEBS Lett 2002, 531(2):299-303.
6. Suzuki T, Namba K, Tsuga H, Nakata H: Regulation of pharmacology by hetero-oligomerization between A_{1} adenosine receptor and P2Y_{2} receptor. Biochem Biophys Res Commun 2006, 351(2):559-565.
7. Fredholm BB, Assender JW, Irenius E, Kodama N, Saito N: Synergistic effects of adenosine A1 and P2Y receptor stimulation on calcium mobilization and PKC translocation in DDT1 MF-2 cells. Cell Mol Neurobiol 2003, 23(3):379-400.
8. Ochisi T, Chen L, Yukawa A, Saitoh Y, Sekino Y, Arai T, Nakata H, Miyamoto H: Cellular localization of adenosine A1 receptors in rat forebrain: immunohistochemical analysis using adenosine A1 receptor-specific monoclonal antibody. J Comp Neurol 1999, 411(2):301-316.
9. Suzuki T, Namba K, Yamagishi R, Kaneko H, Haga T, Nakata H: A highly conserved tryptophan residue in the fourth transmembrane domain of the A_{1} adenosine receptor is essential for ligand binding but not receptor homodimerization. J Neurochem 2009, 110(4):1352-1362.
10. Minneman KP: Heterodimerization and surface localization of G protein coupled receptors. Biochem Pharmacol 2006, 73(8):1043-1050.
11. Bulenger S: Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. Trends Pharmacol Sci 2005, 26(3):131-137.
12. Tonazzini I, Trincavelli ML, Storm-Mathisen J, Martini C, Bergersen LH: Co-localization and functional cross-talk between A_{1} and P2Y, purine receptors in rat hippocampus. Eur J Neurosci 2007, 26(4):890-902.
13. Charara A, Galvani A, Kujavima M, Hall RA, Smith Y: An electron microscope immunocytochemical study of GABA(B) R2 receptors in the monkey basal ganglia: a comparative analysis with GABA(A) R1 receptor distribution. J Comp Neurol 2004, 476(1):165-79.
14. Saifulina VF, Afzalov R, Khiroug L, Cherubini E, Giniatullin R: Reactive oxygen species mediate the potentiating effects of ATP on GABAergic synaptic transmission in the immature hippocampus. J Biol Chem 2006, 281(33):23464-23470.

doi:10.1186/1756-0500-3-323
Cite this article as: Namba et al.: Immunogold electron microscopic evidence of in situ formation of homo- and heteromeric purinergic adenosine A_{1} and P2Y_{2} receptors in rat brain. BMC Research Notes 2010 3:323.

Submit your next manuscript to BioMed Central and take full advantage of:
• Convenient online submission
• Thorough peer review
• No space constraints or color figure charges
• Immediate publication on acceptance
• Inclusion in PubMed, CAS, Scopus and Google Scholar
• Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit