

Published in final edited form as:

*J Neurochem.* 2012 April ; 121(2): 228–238. doi:10.1111/j.1471-4159.2012.07700.x.

## Nucleotides released from A $\beta$ <sub>1–42</sub>-treated microglial cells increase cell migration and A $\beta$ <sub>1–42</sub> uptake through P2Y<sub>2</sub> receptor activation

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### Abstract

Amyloid  $\beta$ -protein (A $\beta$ ) deposits in brains of Alzheimer's disease (AD) patients generate proinflammatory cytokines and chemokines that recruit microglial cells to phagocytose A $\beta$ . Nucleotides released from apoptotic cells activate P2Y<sub>2</sub> receptors (P2Y<sub>2</sub>Rs) in macrophages to promote clearance of dead cells. In this study, we investigated the role of P2Y<sub>2</sub>Rs in the phagocytosis and clearance of A $\beta$ . Treatment of mouse primary microglial cells with fibrillar (fA $\beta$ <sub>1–42</sub>) and oligomeric (oA $\beta$ <sub>1–42</sub>)A $\beta$ <sub>1–42</sub> aggregation solutions caused a rapid release of ATP (maximum after 10 min). Furthermore, fA $\beta$ <sub>1–42</sub> and oA $\beta$ <sub>1–42</sub> treatment for 24 h caused an increase in P2Y<sub>2</sub>R gene expression. Treatment with fA $\beta$ <sub>1–42</sub> and oA $\beta$ <sub>1–42</sub> aggregation solutions increased the motility of neighboring microglial cells, a response inhibited by pre-treatment with apyrase, an enzyme that hydrolyzes nucleotides. The P2Y<sub>2</sub>R agonists ATP and UTP caused significant uptake of A $\beta$ <sub>1–42</sub> by microglial cells within 30 min, which reached a maximum within 1 h, but did not increase A $\beta$ <sub>1–42</sub> uptake by primary microglial cells isolated from P2Y<sub>2</sub>R<sup>–/–</sup> mice. Inhibitors of  $\alpha_v$  integrins, Src and Rac decreased UTP-induced A $\beta$ <sub>1–42</sub> uptake, suggesting that these previously identified components of the P2Y<sub>2</sub>R signaling pathway play a role in A $\beta$  phagocytosis by microglial cells. Finally, we found that UTP treatment enhances A $\beta$ <sub>1–42</sub> degradation by microglial cells, but not in cells isolated from P2Y<sub>2</sub>R<sup>–/–</sup> mice. Taken together, our findings suggest that P2Y<sub>2</sub>Rs can activate microglial cells to enhance A $\beta$  clearance and highlight the P2Y<sub>2</sub>R as a therapeutic target in AD.

### Keywords

Alzheimer's disease; beta-amyloid phagocytosis; microglia; migration; nucleotide; P2Y<sub>2</sub> receptor

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The authors declare no conflict of interest.

Alzheimer's disease (AD) is characterized by the progressive accumulation of amyloid  $\beta$ -protein ( $A\beta$ )-containing plaques in the brain that may be related to alterations in the  $A\beta$  clearance pathway (Tanzi & Bertram 2005; Wang *et al.* 2006).  $A\beta$  peptides (39 to 42 amino acids) are produced from proteolysis of the amyloid precursor protein. Under normal conditions,  $A\beta$  peptides are produced and cleared at equivalent rates in both human and mouse brains (Bateman *et al.* 2006). Thus, a moderate decrease in the rate of clearance could lead to an increase in  $A\beta$  plaque deposition in the brain of AD patients.

Microglial cells are resident macrophages in the brain and the primary immune effector cells in the CNS. In AD brain, microglia play a major role in the internalization and degradation of  $A\beta$  (Frackowiak *et al.* 1992; Bolmont *et al.* 2008; Bergfeld & Forrester 1992). Microglia are closely associated with  $A\beta$  plaques and exhibit an activated proinflammatory phenotype (Perlmutter *et al.* 1990; Frautschy, 1998; Zheng, 2010). In addition, the number and size of microglia increase in proportion to the size of plaques (Wegiel *et al.* 2004; Wegiel *et al.* 2003; Wegiel *et al.* 2001). Recent *in vivo* imaging studies demonstrate that local resident microglia rapidly migrate toward new plaques within 1–2 days of their appearance (Bolmont *et al.* 2008; Meyer-Luehmann, 2008). Other studies suggest that  $A\beta$  deposits in AD brain generate proinflammatory cytokines, *e.g.*, interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and chemokines, *e.g.*, IL-8, macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ), and monocyte chemoattractant protein-1 (MCP-1), that recruit microglial cells to phagocytose  $A\beta$  (Walker *et al.* 1995; Fiala *et al.* 1998, Akiyama *et al.* 2000).

Extracellular nucleotides are released from injured or stressed cells and tissues (Bergfeld & Forrester 1992; Ciccarelli *et al.* 1999; Bodin & Burnstock 2001; Pedersen *et al.* 1999) whereupon they activate cell surface P2 receptors belonging to two structurally distinct families, the G protein-coupled P2Y receptors (P2YRs) and the ion channel P2X receptors (P2XR). P2Y<sub>2</sub>R expression is upregulated in response to stress and injury in a variety of tissues (Koshiba *et al.* 1997; Seye *et al.* 1997; Turner *et al.* 1998; Seye *et al.* 2002) and P2Y<sub>2</sub>R activation increases migration of microglial cells, primary rat cortical astrocytes, arterial smooth muscle cells and endothelial cells (Blondel *et al.* 2000; Honda *et al.* 2001; Chaulet *et al.* 2001; Pillois *et al.* 2002; Kaczmarek *et al.* 2005; Wang *et al.* 2005; Bagchi *et al.* 2005). Recent studies have shown that nucleotides released from apoptotic thymocytes act as “a find-me signal” and enhance phagocytosis of dead cells by macrophages through activation of P2Y<sub>2</sub>R (Elliott *et al.* 2009). Thus, it is plausible that P2Y<sub>2</sub>R activation by nucleotides, such as ATP or UTP, can increase  $A\beta$  phagocytosis by microglial cells in AD brain. In this study, we present results indicating that fibrillar  $A\beta_{1-42}$  (f $A\beta_{1-42}$ ) or oligomeric  $A\beta_{1-42}$  (o $A\beta_{1-42}$ ) aggregates promote the release of nucleotides from primary mouse microglial cells, which enhances cell migration and promotes  $A\beta_{1-42}$  phagocytosis through activation of the P2Y<sub>2</sub>R.

## Methods

### Materials

Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM), penicillin (100 units/ml), and streptomycin (100 units/ml) were obtained from Gibco-BRL (Carlsbad, CA). Anti-integrin  $\alpha_v\beta_5$  (clone P1F6) antibody was purchased from Millipore (Billerica, MA). Pyrazole pyrimidine-type 2 (PP2), NSC23766, LY294002, RO 31-8220, and AG1478 were from Calbiochem (Gibbstown, NJ). TNF- $\alpha$  protease inhibitor-2 (TAPI-2) was from Peptides International (Louisville, KY) and C3 (1  $\mu$ g/ml) was from Cytoskeleton (Denver, CO).  $A\beta_{1-42}$  or scrambled  $A\beta_{1-42}$  lyophilized powder was from American Peptide Company (Sunnyvale, CA). Nucleotides and all other biochemical reagents, including Y27632 and anti-IgG antibody were obtained from Sigma Chemical Co. (St. Louis, MO).

### Primary microglial cell preparation

Primary microglial cells were isolated from inbred neonatal C57BL/6 mice (wild type) and mice deleted of the P2Y<sub>2</sub> receptor on a C57BL/6 background (P2Y<sub>2</sub>R<sup>-/-</sup> mice, strain B6.129P2-P2ry2<sup>tm1Bhk/J</sup>; Jackson Laboratory, Bar Harbor, Maine). The homozygous P2Y<sub>2</sub>R<sup>-/-</sup> mice are viable, fertile, normal in size and do not manifest any physical abnormalities or behavioral deficits. All animals were handled using protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Missouri (protocol # 6728). Briefly, age matched litters of both sexes (postnatal day 2–5) were killed, and brains were collected under aseptic conditions and carefully freed of blood vessels and meninges. Tissues were minced, suspended in trypsin-EDTA (Hyclone) and incubated at 37°C for 20 min. Subsequently, cells were resuspended in Complete Medium comprised of DMEM, 10% FBS, 4.5 g/L glucose, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µg/ml fungizone, OPI supplement (*i.e.*, oxaloacetic acid, pyruvate, insulin; Sigma), and 0.5 ng/ml recombinant mouse GM-CSF (Invitrogen, Carlsbad, CA, Cat # PMC2015). The cell suspension was filtered through a 70 µm cell strainer (Fisher Scientific), and cells were pelleted by centrifugation, resuspended in Complete Medium, and seeded into 75 cm<sup>2</sup> flasks. After 7–10 days, the confluent microglial cell layer was separated from the underlying astrocytic monolayer by gently shaking the flasks overnight at 200 rpm at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Microglia were collected by resuspending the pellet, plated in Complete Medium without GM-CSF, and used for experiments. Microglial cell cultures were routinely greater than 95% pure, as determined by immunohistochemical staining with anti-CD11b (Abcam, Cambridge, CA) and anti-GFAP antibodies (BD Transduction Laboratories, Franklin Lakes, NJ) to identify microglia and astrocytes, respectively.

### Preparation of fibrillar Aβ<sub>1-42</sub> (fAβ<sub>1-42</sub>) and oligomeric Aβ<sub>1-42</sub> (oAβ<sub>1-42</sub>) aggregation solutions

Aβ<sub>1-42</sub> lyophilized powder was dissolved to 1 mg/200 µl in 100% hexafluoroisopropanol and aliquoted into 10 µl portions, dried in a vacuum centrifuge, and stored at -20°C. The fAβ<sub>1-42</sub> and oAβ<sub>1-42</sub> were prepared as described (Dahlgren *et al.* 2002; Stine *et al.* 2003) with slight modification for fAβ<sub>1-42</sub>. For fAβ<sub>1-42</sub>, lyophilized peptides were resuspended in 2 µl DMSO followed by 98 µl PBS and incubated at 37°C for 24 h. A solution of monomeric Aβ<sub>1-42</sub> was prepared by resuspending lyophilized peptide in 2 µl DMSO followed by 98 µl PBS, and the freshly reconstituted Aβ<sub>1-42</sub> solution was used for cell treatment. Western blot analysis indicated that more than 70% of the fAβ<sub>1-42</sub> preparation was converted into large aggregates (Fig. 1A). Dot blot analysis of fAβ<sub>1-42</sub> and oAβ<sub>1-42</sub> suspensions indicated that both preparations reacted with 6E10 (Covance, Princeton, NJ), a monoclonal antibody that recognizes residues 1–16 of Aβ, whereas only the oAβ<sub>1-42</sub> preparation reacted with A11 (Millipore, Billerica, MA), a monoclonal antibody that specifically recognizes oligomeric Aβ (Fig. 1B).

### Dot blot analysis

Five µl of 100 µM fAβ<sub>1-42</sub> and oAβ<sub>1-42</sub> were each spotted on two separate nitrocellulose membranes and allowed to stand for 20 min, followed by blocking with 10% milk in PBS-0.2% Tween 20 (PBST) for 1 h at 4°C. One membrane was incubated with 6E10 antibody (1:5000 dilution), and the other with A11 antibody (1:2000 dilution) for 2 h with gentle shaking at room temperature. Membranes were washed 3X (5 min each wash) with PBST, followed by incubation for 1 h with horseradish peroxidase-conjugated, anti-mouse IgG (Santa Cruz Biotechnology Inc; 1:1000 dilution). The membranes were washed 3X (5 min each wash) with PBST and then developed using the LumiGlo Chemiluminescence System (New England BioLabs, Ipswich, MA), according to the manufacturer's instructions.

### Transmission electron microscopy (TEM)

The fA $\beta$ <sub>1-42</sub> and oA $\beta$ <sub>1-42</sub> (1 $\mu$ M) were applied to grids containing carbon film on 200-square mesh copper grids (Electron Microscopy Sciences, Hatfield, PA). Samples were applied on the carbon side and allowed to adsorb for 10 min at room temperature, excess sample was wicked away with tissue wipe, washed 3X by placing grids sample side down on a droplet of water. Then, heavy metal staining of the samples was done by incubation on a droplet of 2% uranyl acetate for 5–10 min. The excess stain was wicked away with tissue paper and the grids were air dried and visualized with a JEOL 1400 transmission electron microscope operated at 100 kV. The width of A $\beta$  aggregates was measured manually.

### Western blot analysis

Twenty  $\mu$ g of fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates were subjected to 12% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for protein immunoblotting. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature. Then, the membranes were incubated overnight at 4°C with 6E10 antibody that recognizes amino acid residues 1–16 of the A $\beta$  peptide, and the bound antibody was detected with horseradish peroxidase-conjugated rabbit anti-mouse IgG. The membranes were washed and then developed using the LumiGlo Chemiluminescence System (New England BioLabs), according to the manufacturer's instructions.

### Real-time and reverse transcription-PCR analysis of P2Y<sub>2</sub>R mRNA expression

Primary microglial cells were treated with or without 1  $\mu$ M fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates for 24 h and total RNA was isolated and purified using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from 500 ng RNA using the First Strand cDNA Synthesis Kit for RT-PCR (AMV; Roche, Indianapolis, IN). For TaqMan quantitative real-time PCR analysis, the probes (*P2Y<sub>2</sub>R* and *GAPDH*; Applied Biosystems, Carlsbad, California) were labeled at the 5'-end with 6-carboxy-fluorescein phosphoramidite (FAM; for *P2Y<sub>2</sub>R*) or VIC<sup>R</sup> dye (for *GAPDH*; stable endogenous control) and at the 3'-end with minor groove binder (MGB) dye as the quencher. The samples were run in quadruplicate for the *P2Y<sub>2</sub>R* target and the endogenous *GAPDH* control. After computing the relative amounts of *P2Y<sub>2</sub>R* and *GAPDH* for each sample, the data were expressed as a ratio of the amounts of *P2Y<sub>2</sub>R* to *GAPDH*, using Applied Biosystems software. Relative mRNA levels for the control were normalized to 1.

### Extracellular ATP release measurements

Primary microglial cells were incubated for 15 min at 37°C with HEPES buffer (132 mM NaCl, 4 mM KCl, 1.4 mM MgCl<sub>2</sub>, 1.2 mM H<sub>3</sub>PO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 6 mM glucose, 10 mM HEPES, pH 7.4) containing AOPCP, a selective inhibitor of ecto-5'-nucleotidase. Cells were washed 3X, treated with or without 1 $\mu$ M fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates and incubated for different time periods at 37°C. Supernatants were collected at specific time points, and released ATP was measured with the ATP Bioluminescence Assay kit HSII (Roche). ATP levels were calculated based on an ATP standard curve. The results are expressed as ATP released ( $\mu$ mol/L).

### Transwell cell migration assay

Cell migration assays were performed with Transwell cell culture inserts comprised of two chambers separated by an 8- $\mu$ m pore size membrane (Becton Dickinson, Franklin Lakes, NJ). Primary microglial cells ( $1 \times 10^5$ ) suspended in serum-free DMEM were added to the bottom chamber of the inserts placed in a 6-well plate and then treated with solutions of 0.5  $\mu$ M fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates. After 30 min incubation at 37°C, microglial cells ( $1 \times$

$10^5$ ) were added to the upper chamber of the inserts. As a positive control, 100  $\mu$ M ATP or UTP was added to the bottom chamber of two other wells without cells for 30 min followed by addition of cells to the upper chamber of inserts. After 6 h at 37°C, cells remaining on the upper surface of the membrane were removed by scraping with a cotton swab. Cells that migrated through the membrane were fixed with paraformaldehyde, stained with DAPI and counted under an Olympus IX70 inverted microscope at 20X magnification.

### A $\beta$ <sub>1-42</sub> ELISA

Cells were incubated with solutions of 0.5  $\mu$ M fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates in serum-free DMEM for the indicated time, and then washed extensively with PBS and cold acidic buffer (100 mM glycine, 20 mM magnesium acetate, 50 mM KCl, pH 2.2). Washed cells were lysed with Triple Detergent Lysis Buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) containing 5M guanidine hydrochloride. A $\beta$ <sub>1-42</sub> levels in lysates were quantitated using an ELISA (Invitrogen), according to the manufacturer's instructions. All assays were performed in triplicate.

### Lactate dehydrogenase (LDH) cytotoxicity assay

Primary microglial cells ( $1 \times 10^5$  suspended in serum-free DMEM) were plated in 12-well plates and 0.5  $\mu$ M fA $\beta$ <sub>1-42</sub> was added except for control cells. After 24 h, supernatants were collected to measure LDH release from cells and cells were lysed to measure total LDH. The supernatant and cell lysate (50  $\mu$ l) were transferred to 96-well plates, followed by addition of 50  $\mu$ l of CytoTox 96<sup>R</sup> Substrate Mix (Promega, Fitchburg, WI) for 30 min at room temperature in the absence of light followed by addition of Stop Solution (50  $\mu$ l), and sample absorbance was measured at 490 nm. The data are expressed as the amount of LDH released by cells divided by total LDH in cell lysates X 100.

### Statistical analysis

Results are expressed as means  $\pm$  SEM of data obtained from at least 3 experiments. Treatment groups were compared using the t-test. \* $P < 0.05$  or \*\* $p < 0.01$  was accepted as significant.

## Results

### Fibrillar (fA $\beta$ <sub>1-42</sub>) and oligomeric (oA $\beta$ <sub>1-42</sub>) A $\beta$ aggregates induce ATP release in primary microglial cells

Transmission electron microscopy images showed that the fA $\beta$ <sub>1-42</sub> preparations were comprised of fibrils with an average width of 95  $\pm$  18 nm (Fig. 1C). Images for oA $\beta$ <sub>1-42</sub> (Fig. 1D) showed very few globular structures. It is possible that small A $\beta$  aggregates in the solution failed to be trapped by the 200-square mesh grids used for TEM imaging. Incubation of primary microglial cells with solutions of fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates (1  $\mu$ M) increased the release of ATP into the extracellular medium in a time-dependent manner, as compared to the untreated control (Fig. 1E). A peak response was obtained after a 10 min incubation with fA $\beta$ <sub>1-42</sub> (66  $\mu$ M ATP) or oA $\beta$ <sub>1-42</sub> (54  $\mu$ M ATP), aggregation solutions whereupon the levels of extracellular ATP decreased by 1 h. However, ATP levels were detectable up to 2.5 h (data not shown). Solutions of scrambled A $\beta$ <sub>1-42</sub> (scA $\beta$ <sub>1-42</sub>; 1  $\mu$ M) and monomeric A $\beta$ <sub>1-42</sub> (1  $\mu$ M) had no effect on ATP release relative to the untreated control (data not shown). To rule out the possibility that A $\beta$ <sub>1-42</sub> treatment causes extracellular ATP release due to microglial cell lysis, cell supernatants were analyzed for LDH release after fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> treatment, as compared to untreated control. The results showed that a 24 h treatment of wild type and P2Y<sub>2</sub>R<sup>-/-</sup> microglial cells with



fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> did not significantly increase the level of LDH release, as compared to cells incubated without A $\beta$ . (Fig. 1F).

### Incubation with fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates increases P2Y<sub>2</sub>R mRNA expression

Treatment of primary microglial cells with fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates for 24 h caused a 6.5-fold or a 5.2-fold increase in P2Y<sub>2</sub>R mRNA expression, respectively, as compared to untreated control (Fig. 2). Treatment with scA $\beta$ <sub>1-42</sub> under the same conditions had no effect on P2Y<sub>2</sub>R expression.

### fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates cause ATP release, which increases cell migration in primary microglial cells

Previous studies have shown that ATP and UTP can induce cell migration by activating the P2Y<sub>2</sub>R (Seye *et al.*, 2002, Bagchi *et al.*, 2005). Since fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregate treatment induces the release of ATP from primary microglial cells, we tested whether nucleotide release in fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub>-treated microglia can initiate the migration of neighboring microglial cells. A confluent layer of primary microglial cells were seeded on the bottom of a 6-well plate and treated with fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates alone or with apyrase, an enzyme that rapidly hydrolyzes extracellular nucleoside 5'-diphosphates and triphosphates. Additional cells were added to the upper chamber of the Transwell insert and cell migration across the microporous Transwell membrane was measured as described in Methods. We observed that fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates (0.5  $\mu$ M) cause about a 3-fold increase in cell migration across the Transwell membrane, as compared to untreated control (Fig. 3A). Addition of apyrase significantly inhibited the increase in cell migration caused by fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates (Fig. 3A). To verify that nucleotides were capable of inducing migration of microglial cells, ATP or UTP (100  $\mu$ M) was added to the bottom chamber and migration of cells added to the upper chamber was measured. ATP or UTP increased cell migration by ~ 8- or 7-fold, respectively (Fig. 3B). Primary microglial cells isolated from P2Y<sub>2</sub>R<sup>-/-</sup> mice did not migrate in response to fA $\beta$ <sub>1-42</sub>, oA $\beta$ <sub>1-42</sub> or nucleotide treatment (Figs. 3A and 3B), indicating a critical role for the P2Y<sub>2</sub>R and nucleotide release in fA $\beta$ <sub>1-42</sub>-induced cell migration.

### P2Y<sub>2</sub>R activation by UTP or ATP increases fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> uptake by microglial cells

UTP induces A $\beta$ <sub>1-42</sub> uptake with a maximal response obtained at 100  $\mu$ M UTP with an EC<sub>50</sub> of 2.95  $\mu$ M (Fig. 4A). P2Y<sub>2</sub>R activation by UTP or ATP (100  $\mu$ M) increased fA $\beta$ <sub>1-42</sub> uptake in primary microglial cells isolated from wild type mice within 30 min with a peak response occurring within 1 h, as compared to the control (Fig. 4B). UTP or ATP treatment causes significant fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> uptake after 1 h in primary microglial cells, but not in cells isolated from P2Y<sub>2</sub>R<sup>-/-</sup> mice (Fig. 4C), highlighting a role for the P2Y<sub>2</sub>R in A $\beta$ <sub>1-42</sub> uptake by microglial cells.

### The $\alpha_v$ integrin, Rac and Src pathways mediate UTP-induced fA $\beta$ <sub>1-42</sub> uptake

The P2Y<sub>2</sub>R contains an integrin-binding Arg-Gly-Asp (RGD) motif in its first extracellular loop that enables nucleotides to activate integrin signaling pathways (Erb *et al.* 2001). The RGD sequence in the P2Y<sub>2</sub>R promotes its direct interaction with  $\alpha_v\beta_{3/5}$  integrins (Erb *et al.* 2001) that, following P2Y<sub>2</sub>R activation by UTP, mediates an increase in the activation of monomeric G<sub>o</sub> and G<sub>12</sub> proteins and the subsequent stimulation of the small GTPases Rho and Rac (Schenk *et al.* 1999; Bagchi *et al.* 2005; Liao *et al.* 2007). To determine the P2Y<sub>2</sub>R signaling pathways that mediate A $\beta$ <sub>1-42</sub> uptake, we used UTP, a relatively selective ligand for the P2Y<sub>2</sub>R. When primary microglial cells were treated with  $\alpha_v$  integrin neutralizing antibody, UTP-induced A $\beta$ <sub>1-42</sub> uptake was abrogated (Fig. 5A). The selective Src inhibitor, PP2 (1  $\mu$ M), and the selective Rac 1 inhibitor, NSC23766 (50  $\mu$ M) significantly inhibited

UTP-induced A $\beta$ <sub>1-42</sub> uptake (Fig. 5B), whereas C3, an inhibitor of RhoA, Y27632, an inhibitor of ROCK, and specific inhibitors of PI3-kinase, PKC, EGFR tyrosine kinase and matrix metalloproteases (MMPs) failed to inhibit UTP-induced fA $\beta$ <sub>1-42</sub> uptake (Figs. 5B and 5C). These results suggest that UTP-induced P2Y<sub>2</sub>R activation requires  $\alpha_v$  integrins, Src, and Rac to promote A $\beta$ <sub>1-42</sub> uptake.

### P2Y<sub>2</sub>R activation by UTP enhances A $\beta$ <sub>1-42</sub> degradation in microglial cells

To examine the effect of UTP on A $\beta$ <sub>1-42</sub> degradation by microglial cells, we exposed primary microglial cells to fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates (0.5  $\mu$ M) for 1 h to induce uptake and then cells were washed and incubated with or without UTP (100  $\mu$ M) for 24 h. UTP-treated wild type cells showed a significant decrease in the amount of intracellular A $\beta$ <sub>1-42</sub> remaining after 24 h, as compared to cells treated without UTP, in contrast to primary microglial cells from P2Y<sub>2</sub>R<sup>-/-</sup> mice that showed no difference in A $\beta$ <sub>1-42</sub> degradation with or without UTP (Fig. 6). There was virtually no A $\beta$ <sub>1-42</sub> detectable in the cell supernatant over 24 h, indicating that the decrease in intracellular A $\beta$ <sub>1-42</sub> levels was not due to release from cells (data not shown).

## Discussion

Nucleotides, such as ATP and UTP, act as extracellular signaling molecules by activating plasma membrane P2 receptors, including the P2Y<sub>2</sub>R subtype. In this study, we demonstrate the role of the P2Y<sub>2</sub>R in the migration of mouse primary microglial cells and their uptake of fA $\beta$ <sub>1-42</sub> and oA $\beta$ <sub>1-42</sub>. Previous studies (Kim *et al.* 2007; Sanz *et al.* 2009) and data presented here (Fig. 1E) indicate that A $\beta$  treatment stimulates ATP release in microglial cells. A $\beta$ -induced ATP release has been shown to occur via activation of an ATP-gated ion channel, the P2X7 receptor, through interactions with a pannexin hemi-channel (Kim *et al.* 2007; Sanz *et al.* 2009). In this study, we show that fA $\beta$ <sub>1-42</sub> and oA $\beta$ <sub>1-42</sub> aggregates induce significant release of extracellular ATP (Fig. 1E) and an upregulation of P2Y<sub>2</sub>R mRNA (Fig. 2) in mouse primary microglial cells.

The P2Y<sub>2</sub>R has been shown to be upregulated in response to stress and injury in a variety of tissues (Koshiba *et al.* 1997; Seye *et al.* 1997; Turner *et al.* 1998). The P2Y<sub>2</sub>R can be activated equipotently by ATP and UTP and mediates proinflammatory responses, such as the increased cell migration (Chaulet *et al.* 2001; Pillois *et al.* 2002; Bagchi *et al.* 2005), including in microglial cells (Blondel *et al.*, 2000, Honda *et al.*, 2001), and enhanced phagocytic clearance (Elliott *et al.* 2009). In this study, we found that release of > 50  $\mu$ M ATP is induced by fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> in primary microglial cells (Fig. 1E) and, therefore, we routinely used 100  $\mu$ M ATP or UTP for our experiments, since this concentration maximally activates the P2Y<sub>2</sub>R (Bagchi *et al.* 2005; Camden *et al.* 2005; Kong *et al.* 2009). In particular, the use of UTP avoids complications involved with the co-activation of P2X and other P2Y receptors for which ATP, but not UTP, are agonists. Moreover, ATP degradation by cells can generate adenosine and, therefore, the use of UTP also can prevent potential contributions from P1 adenosine receptors.

Chronic inflammation contributes to neurodegeneration in AD (Akiyama *et al.* 2000; Ho *et al.* 2005; Griffin 2006), however, acute inflammation is important for tissue repair and can limit brain damage (Monsonogo & Weiner 2003), in part by promoting the clearance of A $\beta$ . In the AD brain, microglial cells are involved in the inflammatory process through their role in internalization and degradation of A $\beta$  (Frackowiak *et al.* 1992; Bolmont *et al.* 2008). In addition, microglial cells present at A $\beta$  lesions produce several chemotactic agents, including MCP-1 and IL-1 $\beta$  (Walker *et al.* 1995; Fiala *et al.* 1998; Akiyama *et al.* 2000), which promote further microglial cell recruitment. We have shown that microglial cells treated with fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates exhibited increased P2Y<sub>2</sub>R gene expression

within 24 h (Fig. 2). In addition, fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> treatment of wild type mouse primary microglial cells, but not primary microglial cells from P2Y<sub>2</sub>R<sup>-/-</sup> mice, induced an increase in cell migration, which was inhibited by apyrase treatment (Fig. 3A), indicating that nucleotide release activates P2Y<sub>2</sub>Rs in microglial cells to facilitate their accumulation around A $\beta$  aggregates. The effect on cell migration due to nucleotides released from microglial cells treated with solutions of fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> preparations (Fig. 3A) was somewhat weaker than the effect of direct addition of ATP or UTP (Fig. 3B), a difference that was likely due to increased cell-associated nucleotide hydrolysis when ATP was released from cells, although antagonistic contributions of P1 or other P2 receptors cannot be excluded. In this study, ATP release from A $\beta$ <sub>1-42</sub>-treated cells was detectable after 2.5 h (data not shown). *In vivo*, ATP released from microglia likely causes further release of ATP and other factors from surrounding astrocytes that increase migration of microglia (Davalos *et al.* 2005). Although a variety of factors affect the migration of microglial cells (Zola *et al.* 1990; Samuels *et al.* 2010; Fleisher-Berkovich *et al.* 2010; Damani *et al.* 2011), our data indicate that nucleotides increase microglial cell migration through P2Y<sub>2</sub>R activation (Fig. 3). Furthermore, treatment with nucleotides (ATP or UTP) enhanced fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> uptake by primary microglial cells from wild type, but not P2Y<sub>2</sub>R<sup>-/-</sup>, mice (Fig 4C). These results support our hypothesis that P2Y<sub>2</sub>R activation is a probable mechanism whereby brain microglial cells are recruited to phagocytose A $\beta$  deposits.

Our findings indicate that fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> induces P2Y<sub>2</sub>R upregulation (Fig. 2), that likely occurs through P2X<sub>7</sub>R-mediated IL-1 $\beta$  release (Takenouchi *et al.* 2009; Choi *et al.* 2007; Chakfe *et al.* 2002; Suzuki *et al.* 2004). It also has been reported that P2X<sub>7</sub>R activation increases P2Y<sub>2</sub>R expression in rat astrocytes (D'Alimonte *et al.* 2007). Our previous studies have shown that IL-1 $\beta$  upregulates P2Y<sub>2</sub>R expression in neurons, whereupon P2Y<sub>2</sub>R activation promotes non-amyloidogenic APP processing (Kong *et al.* 2009). Therefore, it is plausible that activation of the P2Y<sub>2</sub>R counteracts neurodegenerative aspects of IL-1 $\beta$  by limiting A $\beta$  generation by neurons and by increasing A $\beta$  uptake and degradation by microglia. We postulate that a major effect of P2Y<sub>2</sub>R upregulation in the CNS is to delay the progression of neurodegeneration that occurs with chronic inflammation. A better understanding of the mechanisms responsible for microglial cell recruitment may provide insight into the regulation of the transition from acute to chronic inflammation and neurodegeneration. Our recent data show a 3–4 fold increase in levels of TNF- $\alpha$  and IL-1 $\beta$  release when primary microglial cells were exposed to oA $\beta$ <sub>1-42</sub> for 24 h as compared to control cells without A $\beta$ <sub>1-42</sub> treatment (data not shown). In addition, there was a significant reduction in cytokine release in response to A $\beta$ <sub>1-42</sub> in microglial cells from P2Y<sub>2</sub>R<sup>-/-</sup> mice (data not shown). Further investigations are needed to evaluate the coordinated regulation of nucleotide and cytokine release by microglia in response to A $\beta$ <sub>1-42</sub>.

We reported previously that the G<sub>q</sub>-coupled P2Y<sub>2</sub>R can activate several intracellular signal transduction pathways independent of G<sub>q $\alpha$</sub> -dependent stimulation of PLC that results in intracellular calcium mobilization and PKC activation (Lustig *et al.* 1992; Weisman *et al.* 1999). We also found that an Arg-Gly-Asp (RGD) motif in the first extracellular loop of the P2Y<sub>2</sub>R interacts with  $\alpha_v\beta_{3/5}$  integrins and the integrin-associated protein CD47 to mediate nucleotide-induced cytoskeletal rearrangements and cell migration by enabling the P2Y<sub>2</sub>R to access pools of G<sub>0</sub> and G<sub>12</sub> proteins associated with these integrins, thereby activating Rac1 and RhoA GTPases that regulate actin polymerization (Erb *et al.* 2001; Bagchi *et al.* 2005; Liao *et al.* 2007). Our results indicate that P2Y<sub>2</sub>R-mediated uptake of fA $\beta$ <sub>1-42</sub> requires  $\alpha_v$  integrins, Src and Rac (Fig. 5), suggesting a direct role for P2Y<sub>2</sub>R/ $\alpha_v\beta_{3/5}$  integrin interactions in A $\beta$  uptake. A $\beta$  interacts with a multi-component cell surface receptor complex, including the B-class scavenger receptor CD36, the  $\alpha_6\beta_1$  integrin and the integrin-associated protein CD47, to stimulate the phagocytic activity of microglial cells (Knauer *et*



*al.* 1992; Bamberger *et al.* 2003; Koenigsnecht & Landreth 2004). Since the P2Y<sub>2</sub>R and CD36 both interact with CD47 and its associated integrins, it is possible that all of these proteins form a complex that together regulates phagocytosis of A $\beta$  by microglia. Similar to the signaling cascade activated by the P2Y<sub>2</sub>R (Bagchi *et al.* 2005), the interaction of A $\beta$  with CD36 has been shown to initiate a tyrosine kinase-Vav-Rac1-based signaling cascade, resulting in phagosome formation through Rac-dependent actin cytoskeleton reorganization (Wilkinson *et al.* 2006). Taken together, our results suggest that P2Y<sub>2</sub>R activation plays a role in A $\beta$ <sub>1-42</sub> uptake by microglial cells through P2Y<sub>2</sub>R interaction with  $\alpha_v$  integrins that results in activation of Rac1, a signaling protein known to regulate phagocytosis (Majeed *et al.* 2001; Cougoule *et al.* 2006; Wilkinson *et al.* 2006). Finally, we have shown that P2Y<sub>2</sub>R may play a role in degradation of fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates (Fig. 6) although further experiments are needed to determine the mechanism involved in this process.

An interesting finding is that P2Y<sub>2</sub>R activation promotes the uptake of both fA $\beta$ <sub>1-42</sub> and oA $\beta$ <sub>1-42</sub> aggregates. Previous studies have demonstrated that A $\beta$  exists in several conformations, including monomeric, oligomeric, protofibrillar, fibrillar and soluble fibrillar forms that possess distinct toxic and biological activities (Dahlgren *et al.* 2002; Walsh *et al.* 2002; Deshpande *et al.* 2006; Ajit *et al.* 2009). Our results show that fA $\beta$ <sub>1-42</sub> of predominantly fibrillar form (Fig. 1C) and oA $\beta$ <sub>1-42</sub> aggregates containing low levels of dimers and trimers upregulate P2Y<sub>2</sub>R mRNA expression (Fig. 2) and enhance A $\beta$ <sub>1-42</sub> uptake (Fig. 4) in primary microglial cells. Further studies are needed to isolate and identify the specific A $\beta$  conformations that activate the P2Y<sub>2</sub>R to promote A $\beta$  uptake and degradation.

In conclusion, our results suggest that microglial cells exposed to fA $\beta$ <sub>1-42</sub> or oA $\beta$ <sub>1-42</sub> aggregates release ATP which stimulates their migration and uptake of A $\beta$ <sub>1-42</sub> through a pathway involving P2Y<sub>2</sub>R-mediated activation of  $\alpha_v$  integrin, enabling the downstream stimulation of Src and Rac (see Fig. 7). It has been shown that microglia from the APPsw transgenic mouse model of AD are unable to eliminate  $\beta$ -amyloid deposits (Wegiel *et al.* 2001; Wegiel *et al.* 2003; Wegiel *et al.* 2004), suggesting that failure to clear A $\beta$  from the AD brain contributes to neurodegeneration. Thus, the P2Y<sub>2</sub>R may represent a promising target for preventing A $\beta$  plaque accumulation in the AD brain that leads to neurodegeneration.

## Acknowledgments

This work was supported by NIH grant AG018357 and a National Research Foundation of Korea Grant funded by the Korean Government (NRF-2010-013-E00001).

## Abbreviations used

<b>A<math>\beta</math></b>	$\beta$ -amyloid protein
<b>AD</b>	Alzheimer's disease
<b>AOPCP</b>	$\alpha,\beta$ methylene-adenosine diphosphate
<b>CNS</b>	central nervous system
<b>DMEM</b>	Dulbecco's modified Eagle's medium
<b>DMSO</b>	dimethyl sulfoxide
<b>EGFR</b>	epidermal growth factor receptor
<b>fA<math>\beta</math></b>	fibrillar A $\beta$
<b>FBS</b>	fetal bovine serum

<b>GFAP</b>	glial fibrillary acidic protein
<b>GM-CSF</b>	granulocyte macrophage colony stimulating factor
<b>IL-1<math>\beta</math></b>	interleukin-1 $\beta$
<b>LDH</b>	lactate dehydrogenase
<b>MCP-1</b>	monocyte chemoattractant protein-1
<b>MIP-1<math>\beta</math></b>	macrophage inflammatory protein-1 $\beta$
<b>MMP</b>	matrix metalloprotease
<b>PBS</b>	phosphate buffered saline
<b>PI-3 kinase</b>	phosphatidylinositol 3-kinase
<b>PLC</b>	phospholipase C
<b>PKC</b>	protein kinase C
<b>PP2</b>	pyrazole pyrimidine-type 2
<b>P2Y<sub>2</sub>R</b>	P2Y <sub>2</sub> receptor
<b>RGD</b>	Arg-Gly-Asp
<b>ROCK</b>	Rho-associated protein kinase
<b>scA<math>\beta</math></b>	scrambled A $\beta$
<b>SH3</b>	Src-homology-3
<b>TAPI-2</b>	TNF- $\alpha$ protease inhibitor-2
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor- $\alpha$

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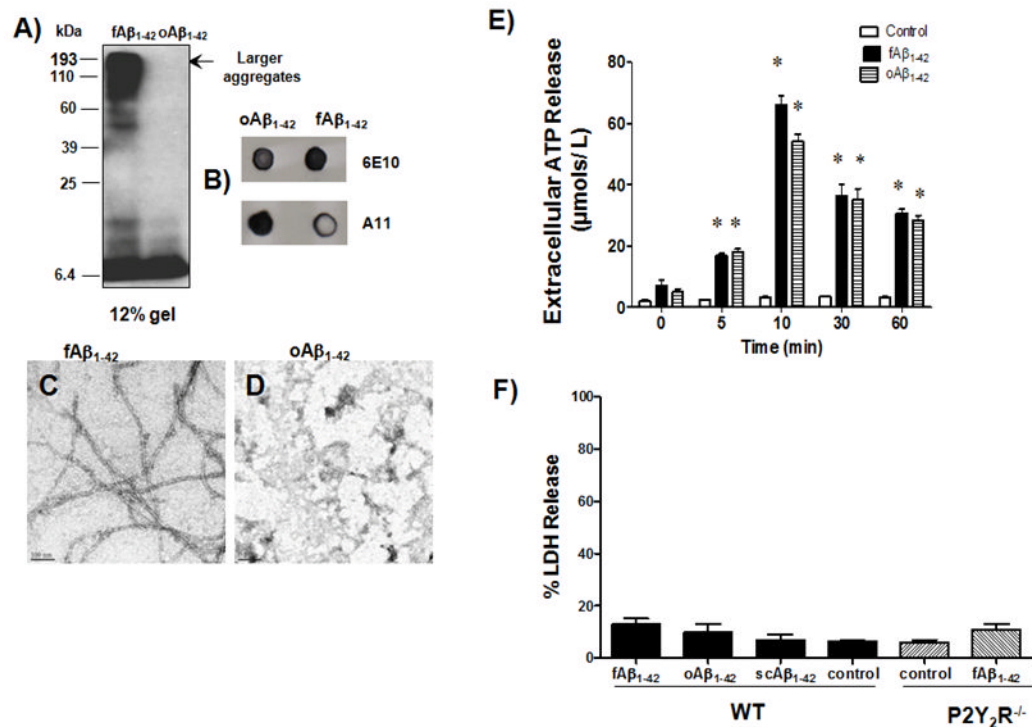
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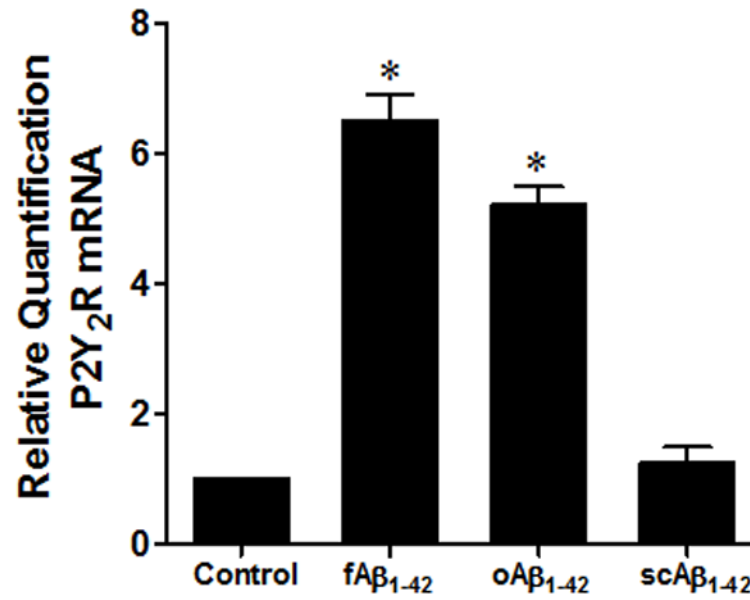
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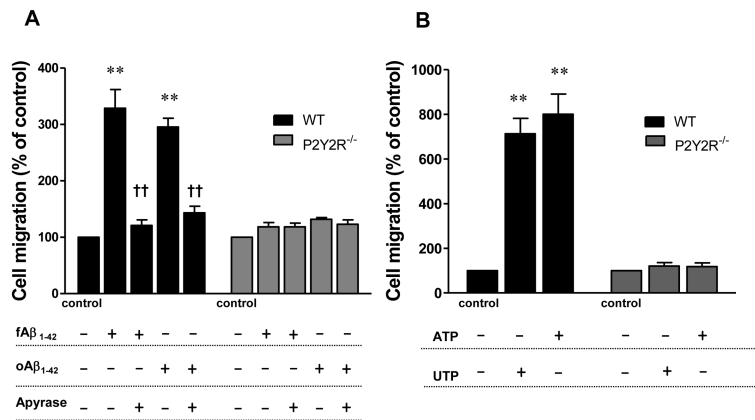
**Fig. 1. fAβ<sub>1-42</sub> and oAβ<sub>1-42</sub> aggregates induce extracellular ATP release in primary microglial cells**

Solutions of fAβ<sub>1-42</sub> and oAβ<sub>1-42</sub> aggregates were prepared as described in Methods. A) Twenty μg of fAβ<sub>1-42</sub> was subjected to 12% SDS-PAGE and Western blot analysis was performed using 6E10 antibody that recognizes amino acid residues 1–16 of the Aβ peptide. B) Five μl of fAβ<sub>1-42</sub> or oAβ<sub>1-42</sub> were spotted on nitrocellulose membrane as described in Methods and probed using 6E10 antibody that recognizes amino acid residues 1–16 of the Aβ peptide and A11 antibody that is specific for oligomer Aβ. C and D) Two μM fAβ<sub>1-42</sub> aggregation solution (C) or oAβ<sub>1-42</sub> aggregation solution (D) was applied on separate carbon-coated copper mesh grids, as described in Methods, and Aβ morphology was analyzed by transmission electron microscopy (TEM); scale bars: 100 nm for fAβ<sub>1-42</sub> and 200 nm for oAβ<sub>1-42</sub>. E) Primary microglial cells were plated at a concentration of  $5 \times 10^5$  cells/well in DMEM and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>. Cells were washed and incubated for 15 min with HEPES buffer containing AOPCP, a selective inhibitor of ecto-5'-nucleotidase, followed by treatment with or without 1 μM fAβ<sub>1-42</sub> or oAβ<sub>1-42</sub> for 5, 10, 30, or 60 min, whereupon ATP release was measured as described in Methods. ATP release with 1 μM monomeric Aβ<sub>1-42</sub> or scaAβ<sub>1-42</sub> was the same as control (data not shown). Data from 3 experiments represent means ± SEM (n = 6), where \**P* < 0.05 indicates a significant difference from untreated controls. F) Primary microglial cells from wild type (WT) or P2Y<sub>2</sub>R<sup>-/-</sup> mice were plated at a concentration of  $1 \times 10^5$  cells/well in DMEM and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 24 h with or without 0.5 μM fAβ<sub>1-42</sub>, oAβ<sub>1-42</sub> or scaAβ<sub>1-42</sub>. Supernatants were collected and LDH release was measured, as described in Methods. Data are averages of triplicate determinations from 3 experiments and differences between each condition were not significant. Statistical analysis (Kruskal-Wallis test) indicated no significant differences between any treatment group (*p* = 0.4060).



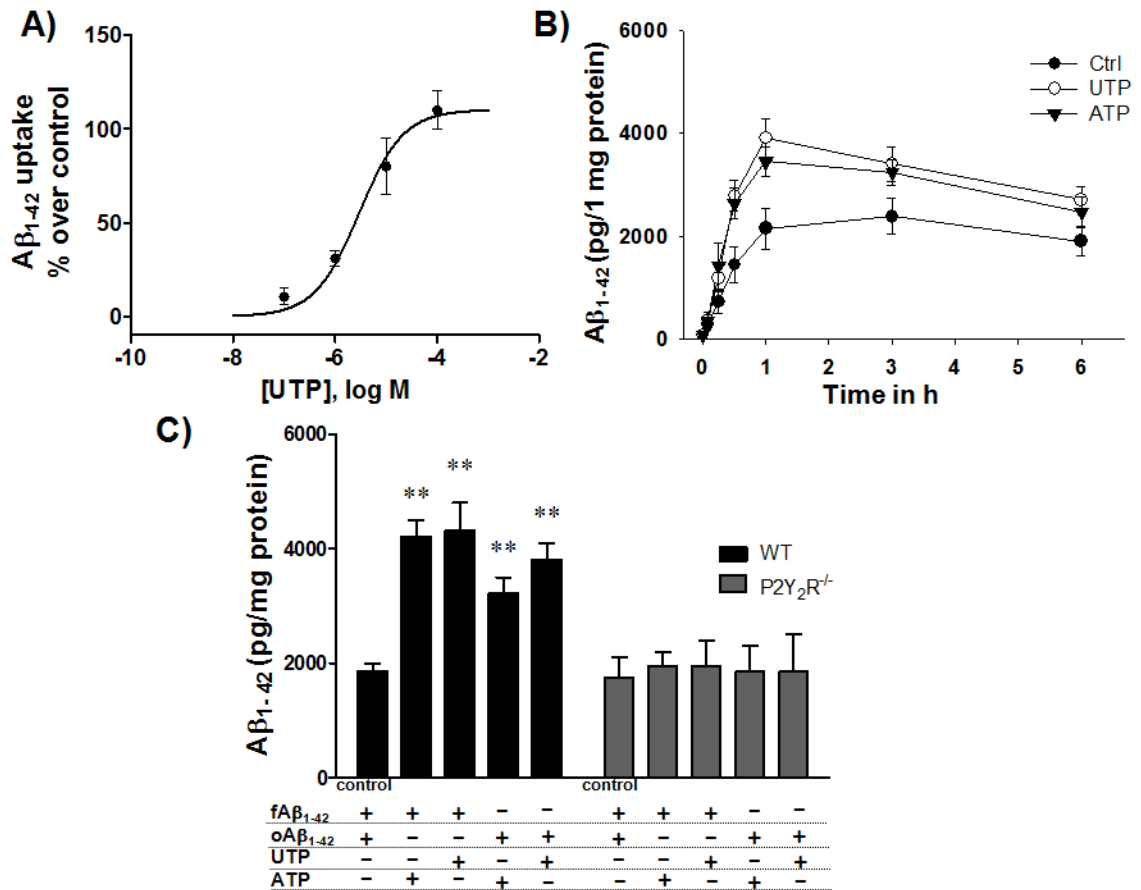
**Fig. 2. fAβ<sub>1-42</sub> and oAβ<sub>1-42</sub> aggregates increase P2Y<sub>2</sub>R mRNA expression**

Primary mouse microglial cells ( $5 \times 10^5$  cells/well) were incubated with or without  $1 \mu\text{M}$  fAβ<sub>1-42</sub>, oAβ<sub>1-42</sub> or scAβ<sub>1-42</sub> aggregation solutions for 24 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Total RNA was isolated from cells using the RNeasy Plus Mini Kit and RT-PCR was performed, as described in Methods. Data from 4 experiments represent means ± SEM (n = 4), where \* $P < 0.05$  indicates a significant difference from untreated controls.



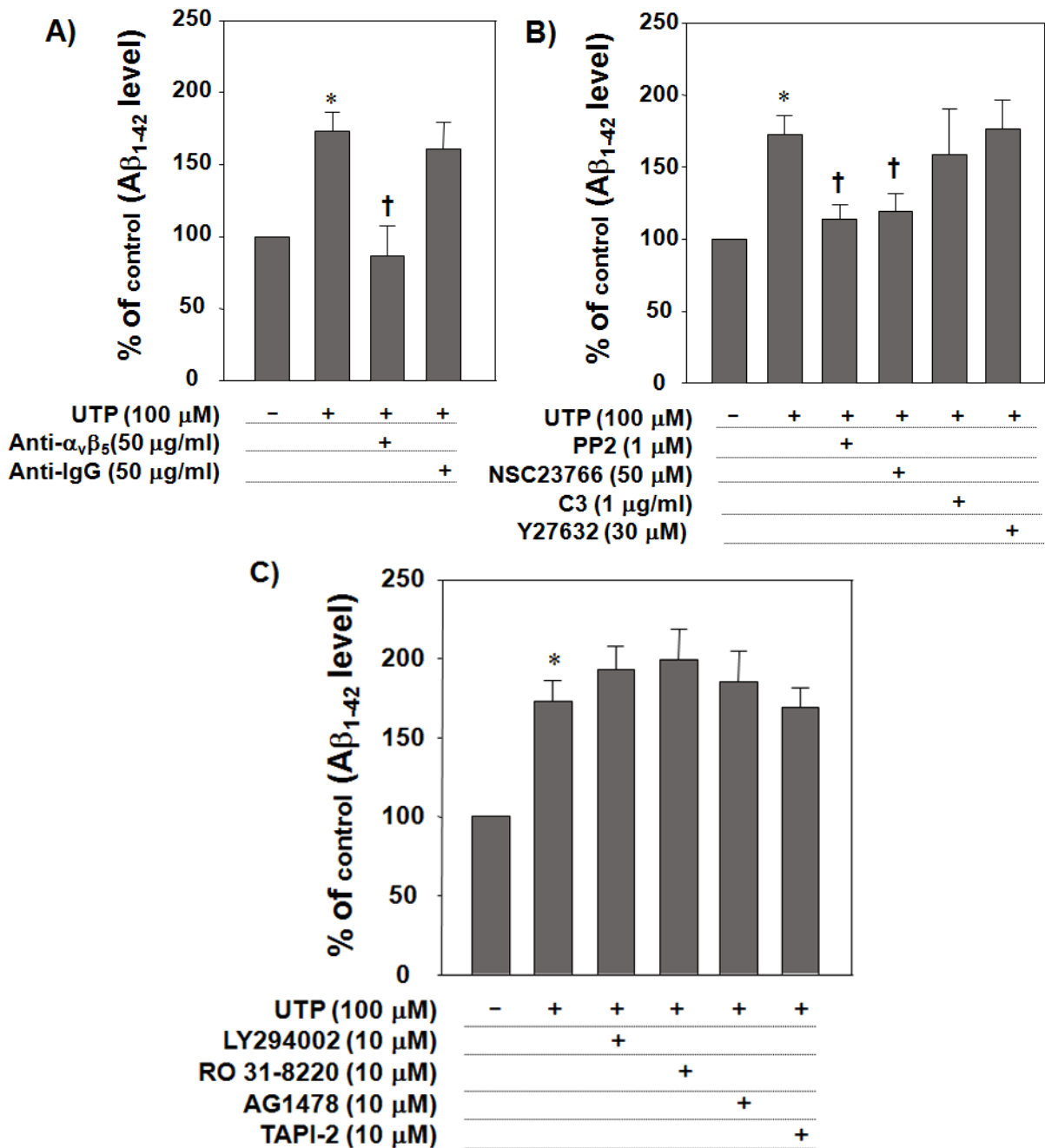
**Fig. 3. ATP released from Aβ<sub>1-42</sub>-treated primary microglial cells induces cell migration**

A) Cells ( $1 \times 10^5$  cells/well) from wild type (WT) or P2Y<sub>2</sub>R<sup>-/-</sup> mice on 6-well plates were pretreated with or without apyrase (5 U/ml) for 30 min and then fAβ<sub>1-42</sub> or oAβ<sub>1-42</sub> (0.5 μM) was added for 30 min. Transwell inserts were placed into the wells and an additional  $1 \times 10^5$  cells/1 ml/well were seeded in the upper chamber of the Transwell insert. B) ATP or UTP (100 μM) was added to the bottom chamber of the Transwell insert in the absence of cells and fAβ<sub>1-42</sub> or oAβ<sub>1-42</sub>, and cells were seeded in the upper chamber. For A) and B) after 6 h, cells on the upper surface of the Transwell membrane were removed and cells that migrated through the membrane were fixed with 5.5% paraformaldehyde and stained with DAPI. The number of cells migrating across the membrane was counted under an Olympus XI70 widefield microscope at 20X magnification (micrographs not shown). Control wells were treated with fAβ<sub>1-42</sub> or oAβ<sub>1-42</sub> and plot represents average of two controls. Data from 3 experiments performed in triplicate represent means ± SEM (n = 6), where \*\**P* < 0.01 indicates a significant difference from untreated controls and ††*P* < 0.01 indicates a significant difference from fAβ<sub>1-42</sub>-treated cells.



**Fig. 4. UTP or ATP increases Aβ<sub>1-42</sub> uptake by microglial cells from wild type but not P2Y<sub>2</sub>R<sup>-/-</sup> mice**

A) Primary microglial cells ( $5 \times 10^5$  cells/well in DMEM) from wild type (WT) mice were incubated in an atmosphere of 5% CO<sub>2</sub> at 37°C and treated with 0.1, 1, 10, or 100 μM UTP and fAβ<sub>1-42</sub> aggregation solution (0.5 μM) for 1 h. Cells were collected and intracellular Aβ<sub>1-42</sub> levels were determined by ELISA. Data represent means ± SEM (n = 3). B) Primary microglial cells ( $5 \times 10^5$  cells/well in DMEM) from WT mice were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> and treated with or without 100 μM UTP or ATP and fAβ<sub>1-42</sub> aggregation solution (0.5 μM) for up to 6 h. Cells were collected 15 min, 1 h, 3 h and 6 h after addition of fAβ<sub>1-42</sub> aggregation solution and intracellular Aβ<sub>1-42</sub> levels were determined by ELISA. C) Primary microglial cells ( $5 \times 10^5$  cells/well in DMEM) from WT or P2Y<sub>2</sub>R<sup>-/-</sup> mice were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> and treated with UTP or ATP and fAβ<sub>1-42</sub> or oAβ<sub>1-42</sub> for 1 h. Control cells were treated with fAβ<sub>1-42</sub> or oAβ<sub>1-42</sub> and the graph represents the average value. Data from 3 experiments represent means ± SEM (n = 3), where \*\*P < 0.01 indicates a significant difference from control.

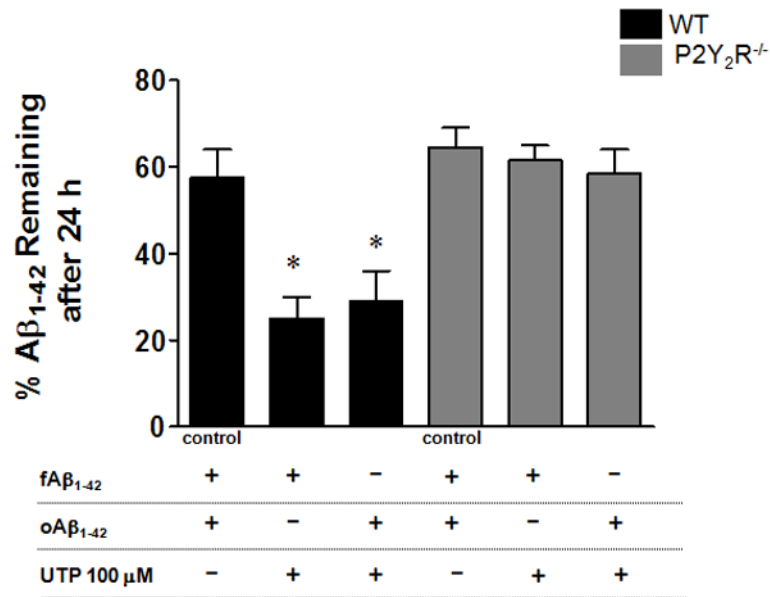


**Fig. 5.  $\alpha_v$  Integrin, Src and Rac mediate UTP-induced  $fA\beta_{1-42}$  uptake**

Primary microglial cells ( $5 \times 10^5$  cells/well in DMEM) were incubated at  $37^\circ\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  and pretreated with (A) anti- $\alpha_v$  neutralizing antibody (50  $\mu\text{g/ml}$ ) or anti-IgG1 antibody (50  $\mu\text{g/ml}$ ), as a negative control, (B) PP2 (a Src inhibitor; 10 $\mu\text{M}$ ), NSC23766 (a Rac1 inhibitor; 50  $\mu\text{M}$ ), C3 (a cell permeable Rho inhibitor; 1 $\mu\text{g/ml}$ ), Y27632 (a ROCK inhibitor; 30  $\mu\text{M}$ ), or (C) LY294002 (a PI-3 kinase inhibitor; 10  $\mu\text{M}$ ), RO 31-8220 (a PKC inhibitor; 10  $\mu\text{M}$ ), AG1478 (an EGFR tyrosine kinase inhibitor; 10  $\mu\text{M}$ ) or TAPI-2 (a MMP inhibitor; 10  $\mu\text{M}$ ) for 30 min and then stimulated with 100  $\mu\text{M}$  UTP in the presence of  $fA\beta_{1-42}$  (0.5 $\mu\text{M}$ ). After 1 h, cells were lysed and intracellular  $A\beta_{1-42}$  levels were determined by ELISA. Data from 3 experiments performed in triplicate represent

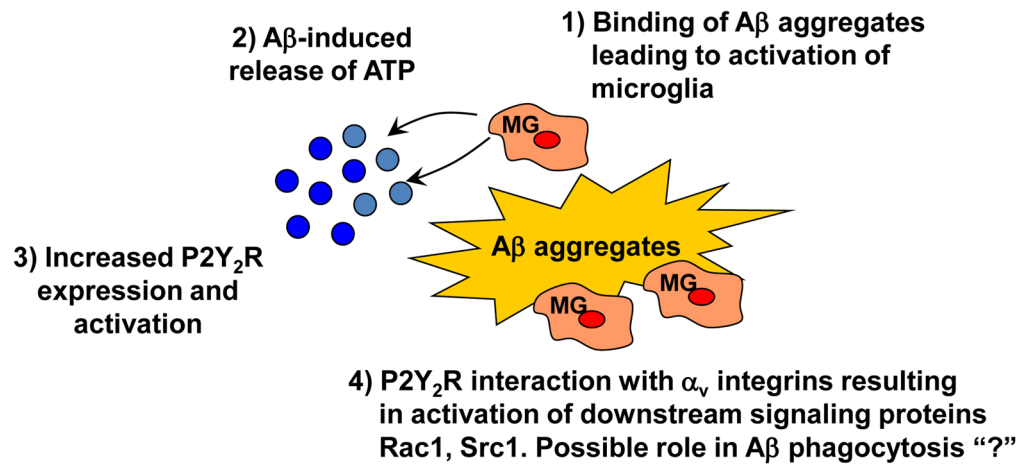


means  $\pm$  SEM (n = 6), where \* $P < 0.05$  indicates a significant difference from untreated control and † $P < 0.05$  indicates a significant difference from UTP treatment alone.



**Fig. 6. UTP enhances Aβ<sub>1-42</sub> degradation by microglial cells from wild type but not P2Y<sub>2</sub>R<sup>-/-</sup> mice**

Primary microglial cells ( $5 \times 10^5$  cells/well in DMEM) from wild type (WT) or P2Y<sub>2</sub>R<sup>-/-</sup> mice were treated with fAβ<sub>1-42</sub> or oAβ<sub>1-42</sub> (0.5 μM) for 1 h, and then the medium was removed and cells were washed with PBS and cold acidic buffer, as described in the Methods. Then, fresh medium was added and cells were treated with or without UTP (100 μM). After 24 h, levels of Aβ<sub>1-42</sub> in cell lysates and secreted Aβ<sub>1-42</sub> in the media were analyzed by ELISA. Control cells were treated with fAβ<sub>1-42</sub> or oAβ<sub>1-42</sub> and data represent the average value. Data represent means  $\pm$  SEM (n = 4), where \*p < 0.05 indicates a significant difference from UTP-untreated control.



**Fig. 7. Schematic representation of the proposed pathway for P2Y<sub>2</sub>R-mediated A $\beta$ <sub>1-42</sub> uptake** Binding of A $\beta$ <sub>1-42</sub> to microglial cells (1) causes ATP release (2) and A $\beta$ -induced upregulation of P2Y<sub>2</sub>R expression and ATP-induced P2Y<sub>2</sub>R activation (3). P2Y<sub>2</sub>R activation regulates A $\beta$ <sub>1-42</sub> uptake by microglial cells through P2Y<sub>2</sub>R interaction with  $\alpha_v$  integrins and perhaps other proteins that results in activation of the downstream signaling molecules Rac1 and Src1 (4).