

# Protein Interacting C-Kinase 1 Modulates Surface Expression of P2Y<sub>6</sub> Purinoreceptor, Actin Polymerization and Phagocytosis in Microglia

Jia Zhu<sup>1</sup> · Zhen Wang<sup>2,4</sup> · Nan Zhang<sup>3</sup> · Jiao Ma<sup>3</sup> · Shui-Lin Xu<sup>1</sup> · Yin Wang<sup>3</sup> · Ying Shen<sup>4</sup> · Yun-Hong Li<sup>3</sup>

Received: 22 April 2015/Revised: 16 October 2015/Accepted: 29 October 2015/Published online: 13 November 2015  
© Springer Science+Business Media New York 2015

**Abstract** Microglia clean up dead cells and debris through phagocytosis in the central nervous system. UDP-activated P2Y<sub>6</sub> receptors (P2Y<sub>6</sub>Rs) induce the formation of phagocytic cup-like structure and P2Y<sub>6</sub>R expression is increased during the phagocytosis. However, it remains unclear how surface expression of P2Y<sub>6</sub>R is increased. PICK1 (protein interacting with C-kinase-1) interacts with various neurotransmitter receptors, transporters, and enzymes. We here report that PICK1 might interact with P2Y<sub>6</sub>R. Surface P2Y<sub>6</sub>R was reduced in microglia from PICK1-knockout mice and PICK1-knockdown BV2 cells, which was also confirmed by electrophysiological recordings, showing that P2Y<sub>6</sub>R-mediated current was increased by PICK1 overexpression but was reduced by PICK1-knockdown in BV2 microglia. Finally, PICK1 was

sufficient to affect cytoskeletal aggregation and phagocytosis both in primary microglia and BV2 cells. These results indicate that PICK1 is an important regulator of P2Y<sub>6</sub>R expression and microglial phagocytosis.

**Keywords** PICK1 · P2Y<sub>6</sub> receptor · BV2 · Microglia

## Abbreviations

AMPA	A-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor
BAR	Bin-Amphiphysin-Rvs
Iba1	Ca <sup>2+</sup> -binding adapter molecule 1
PICK1	Protein interacting with C-kinase 1
PDZ	PSD-95/DlgA/ZO-1
PKC	Protein kinase C
PBS	Phosphate-buffered saline

Jia Zhu and Zhen Wang have contributed equally to this work.

**Electronic supplementary material** The online version of this article (doi:10.1007/s11064-015-1754-3) contains supplementary material, which is available to authorized users.

✉ Jia Zhu  
zhujia0322@163.com

✉ Yun-Hong Li  
976153438@qq.com

- <sup>1</sup> Department of Microbiology and Immunology, Jiaying University School of Medicine, Jiaying, China
- <sup>2</sup> Department of Blood Transfusion, Institute of Molecular Medicine, Zhejiang Provincial People's Hospital, 310014 Hangzhou, China
- <sup>3</sup> Key Laboratory of Craniocerebral Diseases of Ningxia Hui Autonomous Region, Ningxia Medical University, 750004 Yinchuan, China
- <sup>4</sup> Department of Neurobiology, Zhejiang University School of Medicine, 310058 Hangzhou, China

## Introduction

Microglia, the immune cells in the central nervous system (CNS) [1], detect pathological changes in the microenvironment and quickly respond to maintain CNS homeostasis [2]. During CNS development, microglia can swallow excess synapses, thereby regulating the number of synapses [3]. Under pathological conditions, microglia clean up dead cells and debris through the process of phagocytosis, thereby reducing the secondary damage to CNS [4, 5]. Hence, studies on phagocytosis are important to understand roles of microglia in CNS.

It has been shown that receptors on the surface of microglia are remarkably sensitive to subtle changes in microenvironment [6, 7]. Notably, P2Y<sub>6</sub> receptor (P2Y<sub>6</sub>R),

a G-protein-coupled purinergic receptor, is critical to microglial phagocytosis [8, 9]. P2Y<sub>6</sub>R is activated by UDP released by damaged neurons, and surrounds larger external particles through membrane ruffle. Meanwhile, mRNA and protein levels of P2Y<sub>6</sub>R in microglia are also increased, and the phagocytosis can be blocked by P2Y<sub>6</sub>R antagonists [8, 9]. In fact, P2Y<sub>6</sub>R deficiency is closely linked to neurodegenerative and immune diseases in CNS [10]. These findings highlight critical functions of P2Y<sub>6</sub>R in regulating microglial phagocytosis. However, it remains unclear how P2Y<sub>6</sub>R expression is increased and how P2Y<sub>6</sub>R is transported to plasma membrane.

PICK1 (protein interacting with C-kinase-1) is first cloned as a protein kinase C (PKC)-binding protein [11]. It is the only known protein that possesses a PSD-95/DlgA/ZO-1 (PDZ) domain and a Bin-Amphiphysin-Rvs (BAR) domain, and has received considerable attention [12]. Through the PDZ domain, PICK1 interacts with various neurotransmitter receptors, transporters, and enzymes [12, 13]. Through the BAR domain, PICK1 anchors its partners and influences their synaptic localization and function [12, 13]. The best-characterized function of PICK1 is that it regulates trafficking of GluRA2 subunit of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPA) during long-term depression and long-term potentiation [12–16].

In the present work, we found that PICK1 interacted with P2Y<sub>6</sub>R *in vivo* and in cultured microglia and BV2 cells. Overexpression of PICK1 in BV2 increased surface expression of P2Y<sub>6</sub>R and UDP-induced currents. On the contrary, PICK1-knockdown decreased P2Y<sub>6</sub>R currents and surface expression in BV2 cells, which was confirmed by the reduced surface expression of P2Y<sub>6</sub>R in cultured microglia derived from PICK1<sup>-/-</sup> mice. Finally, PICK1 expression was sufficient to change the cytoskeletal arrangement and phagocytosis in microglia. In summary, our work first suggests that PICK1 is an important regulator of P2Y<sub>6</sub>R expression and microglial phagocytosis.

## Experimental and Methods

### Animals

All experiments were approved by the Animal Experimentation Ethics Committees of Jiaying University and Zhejiang University, and were specifically designed to minimize the number of animals used. PICK1-knockout mice were the same as used in previous work [17] and maintained at the Experimental Animal Center of Zhejiang University. Mice were kept under temperature-controlled conditions on a 12:12 h light/dark cycle with food and water *ad libitum*.

### Antibodies and Reagents

Antibodies against P2Y<sub>6</sub>R (#ab92504), P2Y<sub>4</sub>R (#ab180718), Flag (#ab1162), GFP (#ab6556), and GAPDH (#ab181602) were from Abcam (Cambridge, UK). Antibody to flotillin-1 (#61020) was from BD Biosciences (San Jose, CA). Antibody to ionized Ca<sup>2+</sup>-binding adapter molecule 1 (Iba1) (#01919741) was from Wako (Osaka, Japan). Mouse anti-PICK1 antibody (#L20-8) was from NeuroMab (Davis, CA) and rabbit polyclonal anti-PICK1 antibody was from Jun Xia (Hong Kong). Horseradish peroxidase-conjugated secondary antibodies (#31420 and #31460) were from Thermo (Rockford, IL). IgG antibody (#PA5-23094), Alexa Fluor 543-conjugated secondary antibody (#A-11037), Alexa Fluor 488-conjugated secondary antibody (#A-10034), Alexa Fluor 350-conjugated secondary antibody (#A-10039), Alexa Fluor 594-conjugated phalloidin, Alexa Fluor 594 Zymosan A-BioParticles conjugate, and X-rhod-1-AM were from Invitrogen (Carlsbad, CA). The protease inhibitor cocktail was from Merck Chemicals (Darmstadt, Germany). Latruncullin A was from Cayman (Ann Arbor, Michigan). Other chemicals were from Sigma (St. Louis, MO) unless stated otherwise.

### BV2 Microglia Culture

The murine BV2 cell line was maintained in DMEM supplemented with 10 % FBS, 100 U ml<sup>-1</sup> penicillin and 100  $\mu$ g ml<sup>-1</sup> streptomycin at 37 °C in a humidified incubator with 5 % CO<sub>2</sub>. Confluent cultures were passaged by trypsinization. Cells were washed twice with warm DMEM and treated in serum-free medium for at least 4 h before experiments.

### Primary Microglia-Enriched Culture

Microglial cells were prepared from the cortex of P1 mice as described previously [18]. Briefly, cortex was gently triturated and seeded ( $5 \times 10^7$ ) in 150 cm<sup>2</sup> flasks. Two weeks after seeding, microglial cells were separated from astrocytes by shaking the flasks for 5 h at 150 g and were replaced at  $1 \times 10^5$  in a 24-well plate precoated with poly-D-lysine. The enriched microglia were >95 % pure, as determined by immunostaining with antibody against F4/80 (eBioscience, San Diego, CA).

### Lentivirus Transfection

The lentivirus transfection was performed according to previous work [17, 19]. Lentivirus encoding scrambled RNA, PICK1 shRNA, or PICK1 was prepared by GenePharma (Shanghai, China) using entire gene sequence of

rat PICK1 (GenBank ID: AF542094.1), and each RNA was tagged with GFP. The sequence for PICK1 shRNA was 5'-GCC TCA CCA TCA AGA AGT ACC-3' [19]. PICK1 shRNA and scrambled shRNA were driven by U6 promoter, whereas overexpressed PICK1 was driven by elongation factor 1 $\alpha$  promoter. pVSV-G plasmid driven by the CMV promoter expressed envelope glycoprotein of vesicular stomatitis virus and thus replaced the lentiviral env gene. All virus-packed plasmids were transduced into cultures at 20 multiplicities 2 days before experiments. Only when >90 % of cultured cells were transfected, which was confirmed by GFP fluorescence, experiments were continued.

### Surface Biotinylation

Cultured cells were rinsed with cold phosphate-buffered saline (PBS) containing 1 mM MgCl<sub>2</sub> and 0.5 mM CaCl<sub>2</sub>, and incubated in 1 mg/ml sulfo-NHS-LC-biotin (Thermo) for 30 min at 4 °C. Cells were quenched with 100 mM glycine for 15 min at 4 °C, lysed in ice-cold RIPA buffer (50 mM Tris-HCl, 0.1 % SDS, 0.5 % sodium deoxycholate, 1 % Triton-X-100), and centrifuged for 20 min at 4 °C. The BCA assay (Thermo) was used to measure the concentration of proteins in the supernatant, which was next incubated with NeutrAvidin beads (Thermo) for 2 h at 4 °C. Biotinylated proteins were extracted using 2 × SDS sample buffer supplemented with dithiothreitol (50 mM) for 30 min at 50 °C prior to Western blotting.

### Co-Immunoprecipitation (Co-IP)

Co-IP experiments were performed according to previous work [20]. In brief, protein concentration was measured using the BCA assay after centrifugation at 16,000g at 4 °C for 10 min. Supernatant (15  $\mu$ g) was used for input and the remainder was for IP. Pre-cleared solubilized preparations were incubated with anti-PICK1 antibody, which was pre-coupled to protein A-Sepharose beads (GE Healthcare, Waukesha, WI) at 2–4  $\mu$ g antibody/1 ml beads for 2 h in 50 mM Tris-HCl. Proteins on the beads were extracted with 2 × SDS sample buffer and boiled for 5 min before Western blotting.

### Western Blotting

Western blotting was performed as reported previously [20]. In brief, equal amounts of proteins were loaded and fractionated on 10 % sodium dodecyl sulfate-polyacrylamide gels and transferred to PVDF membrane (Millipore, Billerica, MA), immunoblotted with antibodies, and visualized by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). The primary antibody dilutions used

were P2Y<sub>6</sub>R (1:500), P2Y<sub>4</sub>R (1:500), PICK1 (1:2000), flotillin-1 (1:10,000), GAPDH (1:10,000), and secondary antibodies (1:10,000). Film signals were digitally scanned and quantitated using ImageJ 1.42q (NIH, Bethesda, MD).

### Immunocytochemistry

Immunofluorescent staining was performed as described previously [21]. For intracellular staining, cells were fixed 48 h after transfection by 4 % paraformaldehyde and 4 % sucrose in PBS for 15 min, and then permeabilized by 0.2 % Triton X-100 in PBS for 15 min. After blocking with 10 % Bovine Serum Albumin (BSA) for 1 h, transfected BV2 and microglia were incubated with anti-Iba1 antibody (1:500) overnight and another 1-hr incubation with Alexa Fluor 350-conjugated secondary antibody (1:800). For surface staining, transfected BV2 cells were fixed in 4 % paraformaldehyde for 5 min and blocked with BSA for 10 min. These cells were next incubated in anti-Flag antibody (1:500) for 15 min, rinsed for three times with PBS, and incubated with Alexa Fluor 543-conjugated secondary antibody (1:800). To examine F-actin aggregation, Alexa Fluor 594-conjugated phalloidin was added to microglia cultures and BV2 cells. Cells were imaged on a confocal microscope (Olympus, Tokyo, Japan). All parameters used in confocal microscopy were consistent in each experiment, including laser excitation power, detector, and off-set gains. The thickness of the optical slice (pinhole) was adjusted to 0.7  $\mu$ m and z-stacks were acquired at 50 % overlap to detect surface expression of N-terminal Flag-tagged P2Y<sub>6</sub>R, according to previous work [22]. Image J software (NIH, Bethesda, MD) was used to analyze cell images. Imaging data analysts were blind to the experimental conditions until the data were integrated.

### Phagocytosis Assay

The experiment of phagocytosis assay was performed according to previous work [23]. BV2 or microglia cells were incubated with fluorescent Zymosan A (5  $\mu$ g/ml) for 30 min at 37 °C in a humidified incubator with 5 % CO<sub>2</sub>, and then washed with normal medium for several times to remove excess beads. The number of beads per cell was measured for statistics using a confocal microscope (Olympus). At least 20 cells were added to data pool for each experimental condition.

### Electrophysiology

Electrophysiological recordings was performed according to previous work [20, 24]. Cells were placed in a perfusion chamber with continuous gravity flow (2 ml/min) of

Ringer's containing (in mM) 125 NaCl, 2.5 KCl, 1.25  $\text{NaH}_2\text{PO}_4$ , 1  $\text{MgCl}_2$ , 2  $\text{CaCl}_2$ , 26  $\text{NaHCO}_3$  and 25 D-glucose (pH7.4). Recording electrodes (3–5  $\text{M}\Omega$ ) for whole-cell patch clamp contained (in mM) 135 CsMS, 10 CsCl, 10 HEPES, 0.2 EGTA, 4  $\text{Na}_2\text{ATP}$ , and 0.4  $\text{Na}_3\text{GTP}$  (pH 7.3, OSM 300). The final concentration of UDP was 100  $\mu\text{M}$  in the work solution. Cells were voltage-clamped at  $-70$  mV using a 700A amplifier (Molecular Devices, Foster City, CA). The analog signals were sampled at 10 kHz and low-pass filtered at 2 kHz using pClamp 9.0 (Molecular Devices). To measure  $\text{P2Y}_6\text{R}$  currents, a rapid solution changer (RSC-160, Biological Science Instruments, Claix, France) was used to apply drug solutions to recorded cells. The protocol of drug application was controlled by the analog output from the pClamp program.

### $\text{Ca}^{2+}$ Imaging

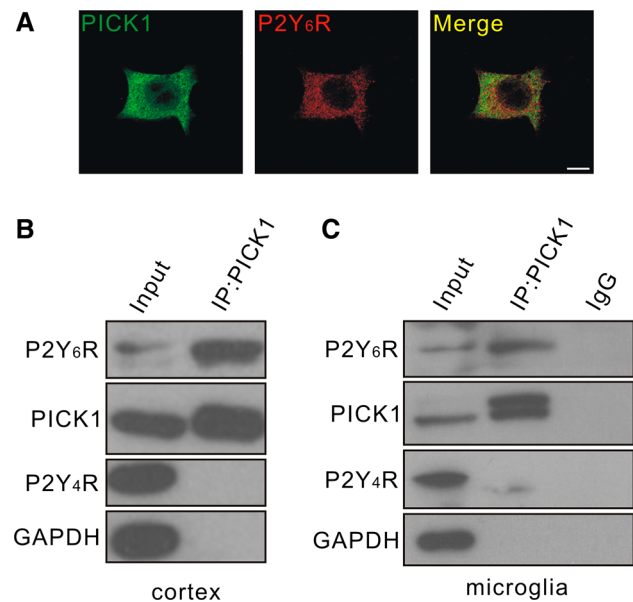
$[\text{Ca}^{2+}]_i$  was determined using  $\text{Ca}^{2+}$ -sensitive dye X-rhod-1-AM according to previous work [25]. BV2 cells were incubated with membrane-permeable X-rhod-1-AM (5  $\mu\text{M}$ ) for 30 min at RT in the dark, and were then transferred to a recording chamber.  $\text{Ca}^{2+}$  transients were elicited by local application of UDP (100  $\mu\text{M}$ ) and recorded using a confocal microscope (Olympus) with the excitation wavelength of 580 nm and the emission wavelength of 645 nm. X-rhod-1 images were sampled in frame-scan mode at a frame rate of 0.1 Hz. For analysis, a region of interest (ROI) that applied to all frames in a series of images was determined by threshold foreground pixels, and these foreground pixels were spatially averaged to calculate  $F$  for each frame. First three frames in each experiment were averaged and used as  $F_0$ . Levels of  $[\text{Ca}^{2+}]_i$  were expressed as  $F/F_0$  ratios.

### Statistical Analysis

Data analysis was performed using Excel 2003 (Microsoft, Seattle, WA), Igor Pro 6.0 (Wavemetrics, Lake Oswego, OR), and SPSS 16.0 statistical program (Chicago, IL). Statistical difference was determined using unpaired two-sided Student's  $t$  test. The accepted level of significance was  $p < 0.05$ . "n" represents the number of cells or cultures tested. Data in the text and figures are presented as mean  $\pm$  SEM.

### Results

The expression of endogenous PICK1 and  $\text{P2Y}_6\text{R}$  were examined in BV2 microglia. As shown by the immunohistochemical staining obtained from confocal microscopy (Fig. 1a), both PICK1 and  $\text{P2Y}_6\text{R}$  were robustly expressed

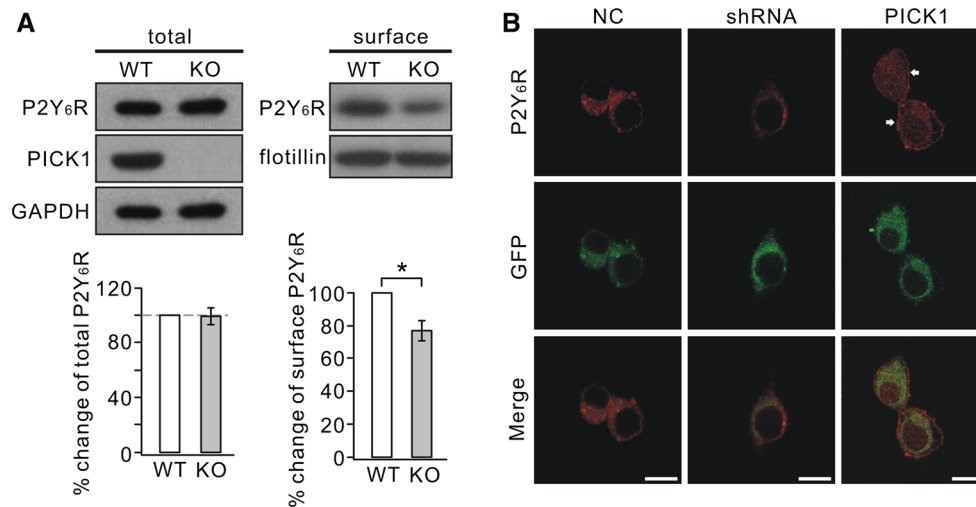


**Fig. 1** PICK1 interacts with  $\text{P2Y}_6\text{R}$ . **a** Representative pictures show the staining of endogenous PICK1 and  $\text{P2Y}_6\text{R}$  in BV2 cells. Scale bar: 10  $\mu\text{m}$ . Note that expression patterns were highly consistent between PICK1 and  $\text{P2Y}_6\text{R}$ . **b** Pre-cleared lysates from mouse cortex were immunoprecipitated with anti-PICK1 antibody. The immunoprecipitates were probed with antibodies to  $\text{P2Y}_6\text{R}$ , PICK1,  $\text{P2Y}_4\text{R}$ , and GAPDH.  $n = 4$ . **c** The immunoprecipitation in pre-cleared lysates from cultured microglial cells using anti-PICK1 antibody. The immunoprecipitates were probed with antibodies to  $\text{P2Y}_6\text{R}$ , PICK1,  $\text{P2Y}_4\text{R}$ , and GAPDH. Mouse IgG was negative control.  $n = 4$

in BV2 cells and displayed a diffuse pattern in the cytosol but not nucleus. Moreover, the distribution pattern of PICK1 was very similar to that of  $\text{P2Y}_6\text{R}$  (Fig. 1a), implying that that PICK1 might interact with  $\text{P2Y}_6\text{R}$ . To investigate the potential interaction between them, Co-IP assay was performed. First, lysates from mouse cortex were immunoprecipitated with PICK1 antibody. We found that endogenous  $\text{P2Y}_6\text{R}$ , but not  $\text{P2Y}_4\text{R}$ , was pulled down by PICK1 when PICK1 was immunoprecipitated (Fig. 1b). Second, Co-IP assay was performed in the primary microglial culture. Likewise, anti-PICK1 antibody pulled down  $\text{P2Y}_6\text{R}$  but not  $\text{P2Y}_4\text{R}$  (Fig. 1c). PICK1 did not pull down IgG, showing the specificity of Co-IP experiments (Fig. 1c). Together, these results suggest that PICK1 may associate with  $\text{P2Y}_6\text{R}$ .

We next investigated whether PICK1 affects the expression of  $\text{P2Y}_6\text{R}$ . Microglial cells from wild-type and PICK1-knockout mice were cultured. Surface biotinylation and Western blotting were used to detect total and surface expressions of  $\text{P2Y}_6\text{R}$  in primary microglia. Our results showed that total expression of  $\text{P2Y}_6\text{R}$  was not changed in PICK1-knockout microglia (Fig. 2a). However, the amount of  $\text{P2Y}_6\text{R}$  on the cell surface was decreased significantly in PICK1-knockout microglia (Fig. 2a), suggesting that





**Fig. 2** PICK1 affects surface expression of P2Y<sub>6</sub>R. **a** P2Y<sub>6</sub>R in the membrane fraction decreased in PICK1-knockout mice (KO) compared to wild-type mice (WT). GAPDH and flotillin were used as loading controls for total and membrane fractions, respectively. Percentage changes of P2Y<sub>6</sub>R expression were 96 ± 6 % (total) and 76 ± 6 % (surface) (n = 4 pairs). **b** BV2 cells were transfected for 48 h with N-terminal Flag-tagged P2Y<sub>6</sub>R together with lentiviral

GFP-tagged scramble RNA (NC), GFP-tagged PICK1-shRNA (shRNA), or GFP-tagged PICK1 (PICK1). After fixation and immunostaining with anti-Flag antibody, fluorescent fusion proteins were visualized under the corresponding channels. Note that surface P2Y<sub>6</sub>R was increased as *white arrows* indicate. Scale bars: 20 μm. \*p < 0.05

PICK1-knockout attenuates P2Y<sub>6</sub>R on the surface of microglia. To further confirm this result, BV2 cells were transfected with plasmid encoding N-terminal Flag-tagged P2Y<sub>6</sub>R, meanwhile they were also transduced with lentiviral GFP-tagged scrambled RNA, GFP-PICK1-shRNA, or GFP-PICK1. As shown in Fig. S1, PICK1-shRNA knockdown effectively suppressed the expression of PICK1 in BV2 cells, consistent with previous work (17, 19). Surface expression of P2Y<sub>6</sub>R was examined 2 days after the transfection using the antibody against Flag. Our results showed that the intensity of Flag was much reduced in PICK1-shRNA group, but was significantly increased in PICK1 group (Fig. 2b). Meanwhile, the fluorescent intensity of tagged-GFP with viral transfection appeared same among three groups (Fig. 2b). These results confirm that surface expression of P2Y<sub>6</sub>R is modulated by PICK1.

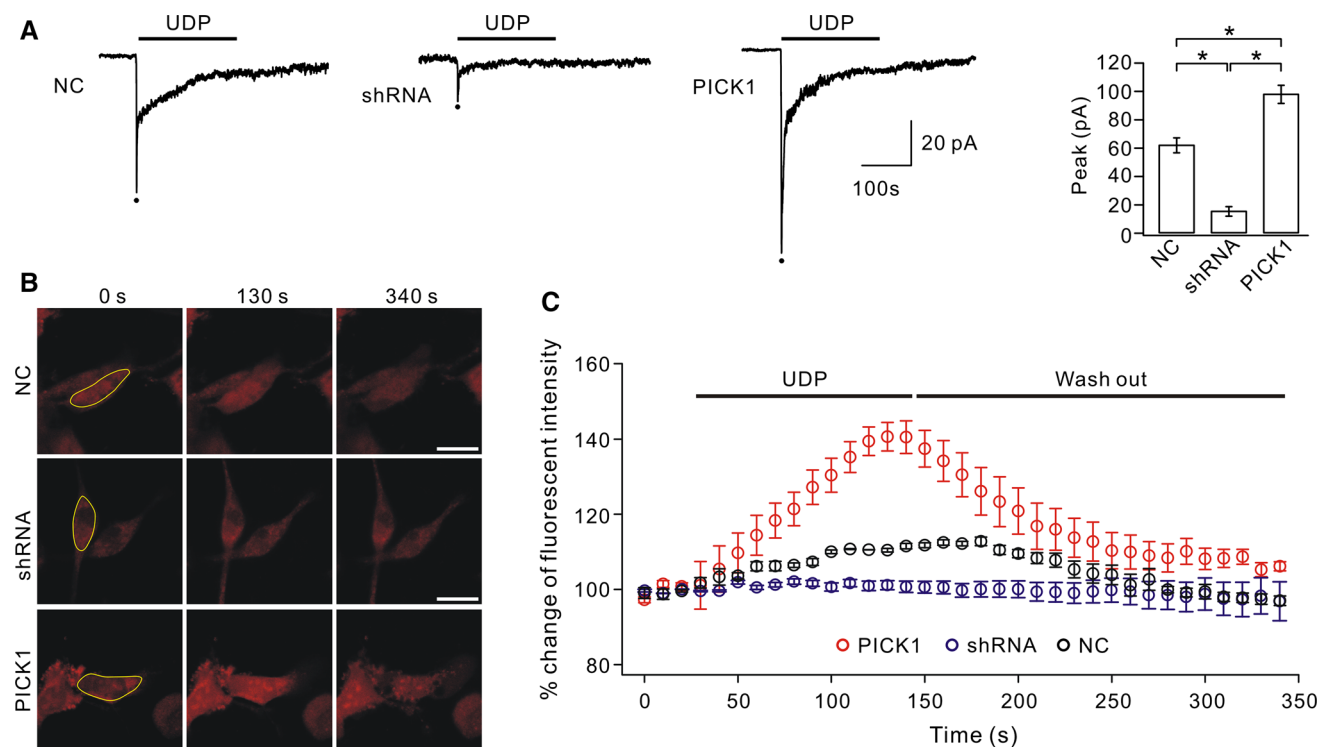
We next investigated whether PICK1 influences the function of P2Y<sub>6</sub>R. BV2 cells were transduced with control lentivirus, PICK1-shRNA, or PICK1, and whole-cell P2Y<sub>6</sub>R currents was evoked by UDP, which was the specific agonist for P2Y<sub>6</sub>R [26]. As shown in Fig. 3a, the peak of P2Y<sub>6</sub> currents was significantly increased by PICK1 overexpression. On contrary, P2Y<sub>6</sub>R current was significantly suppressed by PICK1-knockdown compared to either NC or PICK1 group (Fig. 3a). Since activation of P2Y<sub>6</sub>R leads to an increase in intracellular Ca<sup>2+</sup>, we next investigated whether PICK1 influences P2Y<sub>6</sub>R-mediated [Ca<sup>2+</sup>]<sub>i</sub>. Three different groups of BV2 cells were incubated with a membrane-permeable Ca<sup>2+</sup> indicator, X-rhod-

1-AM (5 μM). Confocal imaging showed that UDP induced an increase in Ca<sup>2+</sup> fluorescence in NC group (Fig. 3b). When BV2 cells were transfected with PICK1-shRNA, UDP application did not induce an obvious increase of [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 3c). When BV2 cells were transfected with PICK1, UDP application induced a remarkable increase of [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 3c). Taken together, these results indicate that PICK1 is essential to P2Y<sub>6</sub>R-mediated currents and Ca<sup>2+</sup> transient.

It has been shown that the activation of P2Y<sub>6</sub>R is sufficient to affect cytoskeletal arrangement and formation of “phagocytic cup” in microglial cells [27]. Given the significance of PICK1 on the activation of P2Y<sub>6</sub>R, we examined if PICK1 is able to affect cytoskeletal arrangement in BV2 cells and microglia. BV2 cells were transduced with control lentivirus or lentiviral plasmid encoding PICK1 and then subjected to UDP stimulation. The pattern of F-actin was indicated by phalloidin staining and the efficiency of transfection was shown by GFP. In control, UDP stimulation (3 min) caused relatively little actin polymerization, shown as concentrated phalloidin staining in the peripheral part of plasma membrane (Fig. 4a). In the condition of PICK1 overexpression, the number of actin aggregation induced by UDP stimulation was markedly increased (Fig. 4b). Similar experiments were conducted in cultured microglia. Likewise, UDP stimulation (3 min) caused less actin polymerization in control microglia (Fig. 4c), whereas the number of actin aggregation was significantly increased in the condition of PICK1

overexpression (Fig. 4d). Taken together, these data indicate that PICK1 is sufficient to facilitate UDP-induced actin aggregation and ruffle formation.

Finally, we investigated whether PICK1 is sufficient to affect phagocytic capacity of BV2 cells and primary microglia. First, BV2 cells were transduced with control lentivirus, PICK1-shRNA, or PICK1, and then presented with fluorescent Zymosan-A microbeads and UDP (100  $\mu$ M) for 30 min [23]. There was no difference in the number of microbeads among three groups before UDP treatment (Fig. S2). However, PICK1-shRNA significantly reduced phagocytic capacity of BV2 cells (shRNA:  $1.2 \pm 0.5$  beads/cell;  $n = 20$  cells) compared to control (NC:  $3.0 \pm 0.5$  beads/cell;  $n = 21$  cells), whereas PICK1 remarkably increased phagocytic capacity of BV2 cells (PICK1:  $5.0 \pm 1.1$  beads/cell;  $n = 21$  cells) (Fig. 5a). Second, primary microglia culture from WT and PICK1-knockout mice were presented with fluorescent Zymosan-A microbeads and UDP (100  $\mu$ M) for 30 min to assay phagocytic capacity [23]. In consistent with BV2 cells, the number of beads in PICK1-knockout microglial cells was

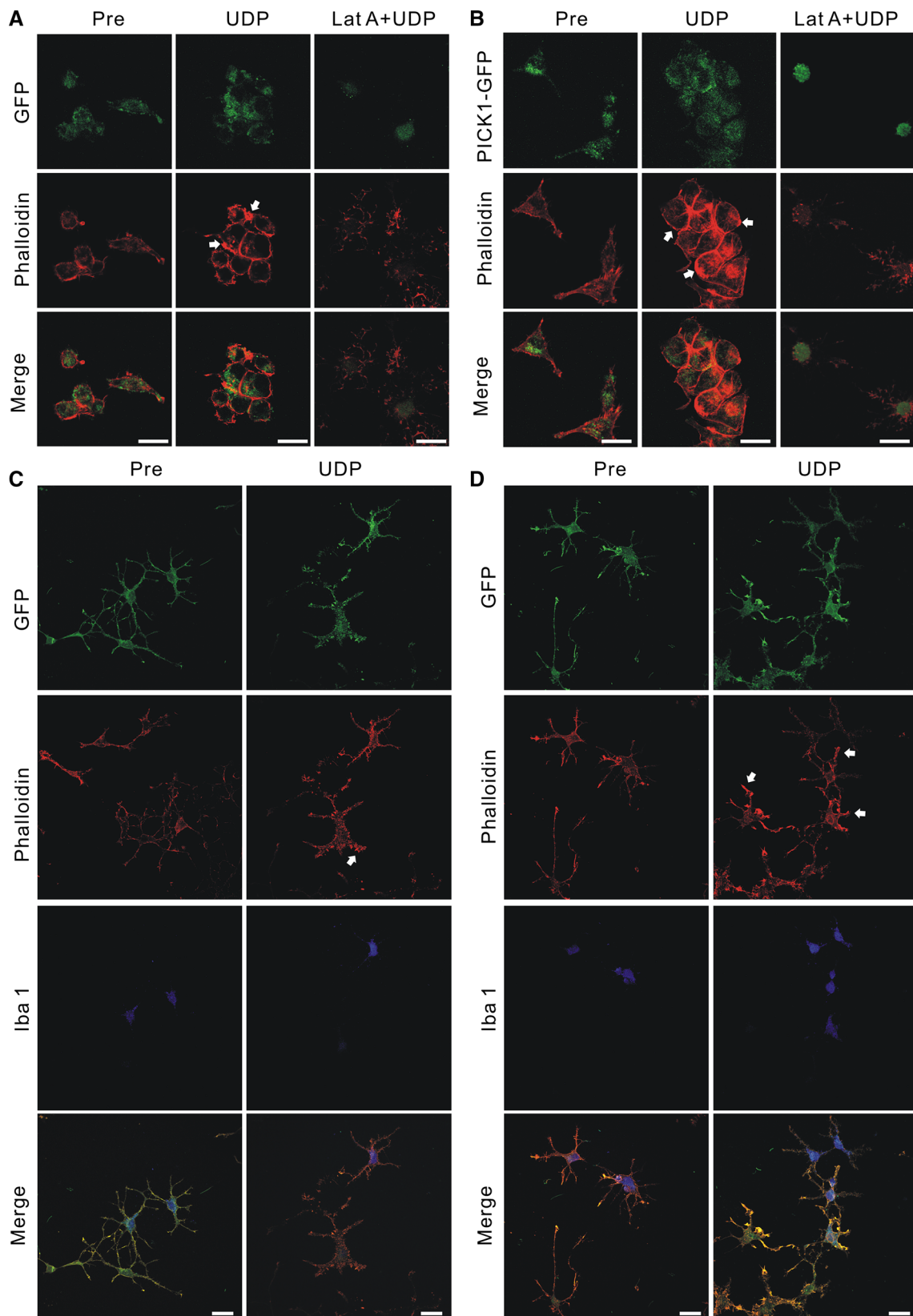


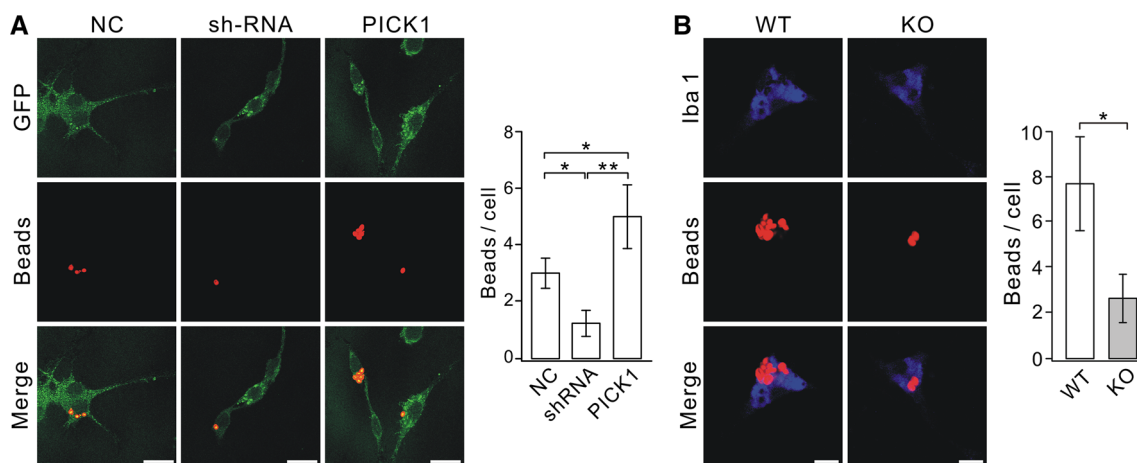
**Fig. 3** PICK1 affects P2Y<sub>6</sub>R-mediated currents and Ca<sup>2+</sup> transient. **a** UDP (100  $\mu$ M) application induced P2Y<sub>6</sub>R-mediated currents in BV2 cells, which were treated with control lentivirus (NC), PICK1-shRNA (shRNA), or overexpressing PICK1 (PICK1). PICK1 enhanced while PICK1-shRNA attenuated UDP-induced currents in BV2 cells. Averaged amplitudes of peak current were  $62 \pm 5$  pA (NC),  $16 \pm 3$  pA (shRNA), and  $98 \pm 6$  pA (PICK1). **b** [Ca<sup>2+</sup>]<sub>i</sub> responses in BV2 cells treated with control lentivirus (NC), PICK1-shRNA (shRNA), or overexpressing PICK1 (PICK1). [Ca<sup>2+</sup>]<sub>i</sub> was

**Fig. 4** PICK1 affects the aggregation of F-actin. **a** UDP induced local actin polymerization. BV2 cells were transfected with GFP-tagged control plasmid (GFP) and stimulated without (Pre) or with 100  $\mu$ M UDP for 3 min. Cells were stained with phalloidin (red). Note that latrunculin A (Lat A) blocked UDP-induced polymerization. White arrows show the aggregation of F-actin on the periphery of BV2 cells. Scale bars: 20  $\mu$ m. **b** BV2 cells were transfected with lentiviral GFP-tagged plasmid encoding PICK1 (PICK1-GFP) and stimulated without (Pre) or with UDP (100  $\mu$ M) for 3 min. UDP induced more F-actin aggregation shown by white arrows. Scale bars: 20  $\mu$ m. **c** Primary microglial cultures were transfected with GFP-tagged control plasmid (GFP), and stimulated without (Pre) or with UDP (100  $\mu$ M) for 3 min. Microglia were stained with Iba1 (blue) and phalloidin (red), showing aggregated F-actin (white arrows). Scale bars: 20  $\mu$ m. **d** Microglia were transfected with lentiviral GFP-tagged plasmid encoding PICK1, and stimulated without (Pre) or with UDP (100  $\mu$ M) for 3 min. More aggregations of F-actin (white arrows) were found in this condition. Scale bars: 20  $\mu$ m (Color figure online)

significantly decreased compared to WT (WT:  $7.7 \pm 2.6$  beads/cell,  $n = 20$  cells; knockout:  $2.1 \pm 1.1$  beads/cell,  $n = 20$  cells) (Fig. 5b). Thus, these results indicate that PICK1 facilitates microglial phagocytic activity.

monitored every 10 s and UDP (100  $\mu$ M) was introduced after 30-s baseline. The same microscopic fields were shown before (0 s) and after (130 s, 340 s) UDP stimulation. Yellow outlines indicate ROI where foreground X-rhod-1 fluorescence was measured. Scale bar: 20  $\mu$ m. **c** Time-course of X-rhod-1 fluorescence ( $F/F_0$ ). The percent changes of intensity of X-rhod-1 fluorescence ( $F/F_0$ ) at  $t = 130$  s were  $110 \pm 1$  % (NC;  $n = 6$ ),  $101 \pm 2$  % (shRNA;  $n = 5$ ), and  $141 \pm 4$  % (PICK1;  $n = 5$ ). \*  $p < 0.05$  (Color figure online)





**Fig. 5** PICK1 affects phagocytic capacity. **a** BV2 cells were transfected with GFP-tagged control lentivirus (NC), PICK1-shRNA (shRNA), or PICK1, and then presented with Zymosan-A microbeads and UDP (100  $\mu$ M). Scale bars: 20  $\mu$ m. Data are pooled from 3

independent experiments. **b** Primary microglia derived from wild-type (WT) and PICK1-knockout (KO) mice were presented with Zymosan-A microbeads and UDP (100  $\mu$ M). Scale bars: 10  $\mu$ m. Data are pooled from 3 independent experiments. \* $p$  < 0.05. \*\* $p$  < 0.01

## Discussion

In the present work, we showed that PICK1 was associated with P2Y<sub>6</sub>R in BV2 cells and cultured microglia. PICK1 might engage in the trafficking of P2Y<sub>6</sub>R, which was suggested that its surface expression (but not total), its-mediated current, and its-mediated Ca<sup>2+</sup> transient were all regulated by PICK1. Importantly, PICK1 was sufficient to enhance actin polymerization to shape filopodia-like protrusions and phagocytosis. In summary, these results indicated that PICK1 may have essential roles in microglial phagocytosis through modulating P2Y<sub>6</sub>R.

Microglia are quite sensitive to brain damage. They are immediately activated in response to brain injury and migrate to the sites of injured cells or their debris [2]. These phenotypes show that microglial phagocytosis is critical to the reconstruction after brain damage and is thereby beneficial to the brain [2]. However, functions of microglia are yet under controversial because over-activated microglia also cause cell loss [2]. It has been shown that P2Y<sub>6</sub>R plays important roles in microglial phagocytosis. For example, UDP released from damaged cells acts as an “eat-us” signal and triggers the phagocytosis through P2Y<sub>6</sub>R [6]. Moreover, P2Y<sub>6</sub>R is activated and its level on the membrane is elevated during the phagocytosis [13, 14]. Despite these findings, it remains unsolved how P2Y<sub>6</sub>R and its activity are regulated during the phagocytosis. In the present work, we added an important signaling to the underlying mechanism for microglial phagocytosis by demonstrating that PICK1, a peripheral protein, affects the targeting of P2Y<sub>6</sub>R to cell surface. Critical evidence for our conclusion includes: (1) Surface expression of P2Y<sub>6</sub>R was significantly increased in BV2 cells when PICK1 was co-

expressed; (2) Number of actin aggregation induced by UDP was markedly increased with PICK1 over-expression in BV2 cells and primary microglia; (3) Phagocytic capacity of BV2 cells and primary microglia was increased when PICK1 was over-expressed but was decreased by PICK1-knockdown or PICK1-knockout.

Although our evidence suggests that PICK1 may affect the trafficking of P2Y<sub>6</sub>R in microglia, how PICK1 increases surface expression of P2Y<sub>6</sub>R is not solved in the present work. Through PDZ domain and BAR domain, PICK1 interacts with receptors, transporters, and enzymes, and thereby participates in many aspects of brain function [12, 13, 28]. We recently show that PICK1 modulates membrane expression of excitatory amino acid transporter 3 (EAAT3) through Rab11-positive recycling endosomes [17]. In addition, PICK1 is shown to help the transport of insulin-containing mature secretory granules to the membrane and facilitate insulin secretion [29]. Thus, it is conceivable that PICK1 is involved in the formation of different trafficking vesicles, which provides a potential mechanism to enriched membrane P2Y<sub>6</sub>R.

## Conclusion

PICK1 interacts with P2Y<sub>6</sub>R in microglia. It thereby regulates the surface expression of P2Y<sub>6</sub>R and its-mediated currents. Moreover, PICK1 modulates the aggregation of F-actin and phagocytosis in BV2 cells and primary microglia.

**Acknowledgments** This work was supported by grants from National Natural Science Foundation of China (81501043, 31460257, 31471024, and 81571098).



## Compliance with Ethical Standards

**Conflict of interest** All authors declare that there are no conflicts of interest on the paper.

## References

- Aloisi F (2001) Immune function of microglia. *Glia* 36:165–179
- Hanisch UK, Kettenmann H (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 10:1387–1394
- Ji K, Akgul G, Wollmuth LP, Tsirka SE (2013) Microglia actively regulate the number of functional synapses. *PLoS One* 8:e56293
- Sierra A, Abiega O, Shahraz A, Neumann H (2013) Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis. *Front Cell Neurosci* 7:6
- Chan A, Hummel V, Weilbach FX, Kieseier BC, Gold R (2006) Phagocytosis of apoptotic inflammatory cells downregulates microglial chemoattractive function and migration of encephalitogenic T cells. *J Neurosci Res* 84:1217–1224
- Koizumi S, Ohsawa K, Inoue K, Kohsaka S (2013) Purinergic receptors in microglia: functional modal shifts of microglia mediated by P2 and P1 receptors. *Glia* 61:47–54
- Mika T, Prochnow N (2012) Functions of connexins and large pore channels on microglial cells: the gates to environment. *Brain Res* 1487:16–24
- Koizumi S, Shigemoto-Mogami Y, Nasu-Tada K, Shinozaki Y, Ohsawa K, Tsuda M, Joshi BV, Jacobson KA, Kohsaka S, Inoue K (2007) UDP acting at P2Y<sub>6</sub> receptors is a mediator of microglial phagocytosis. *Nature* 446:1091–1095
- Inoue K (2007) UDP facilitates microglial phagocytosis through P2Y<sub>6</sub> receptors. *Cell Adh Migr* 1:131–132
- Zhang Z, Wang Z, Ren H, Yue M, Huang K, Gu H, Liu M, Du B, Qian M (2011) P2Y<sub>6</sub> agonist uridine 5'-diphosphate promotes host defense against bacterial infection via monocyte chemoattractant protein-1-mediated monocytes/macrophages recruitment. *J Immunol* 186:5376–5387
- Staudinger J, Zhou J, Burgess R, Elledge SJ, Olson EN (1995) PICK1: a perinuclear binding protein and substrate for protein kinase C isolated by the yeast two-hybrid system. *J Cell Biol* 128:263–271
- Xu J, Xia J (2006) Structure and function of PICK1. *Neurosignals* 15:190–201
- Hanley JG (2008) PICK1: a multi-talented modulator of AMPA receptor trafficking. *Pharmacol Ther* 118:152–160
- Hanley JG, Henley JM (2005) PICK1 is a calcium-sensor for NMDA-induced AMPA receptor trafficking. *EMBO J* 24:3266–3278
- Steinberg JP, Takamiya K, Shen Y, Xia J, Rubio ME, Yu S, Jin W, Thomas GM, Linden DJ, Huganir RL (2006) Targeted in vivo mutations of the AMPA receptor subunit GluR2 and its interacting protein PICK1 eliminate cerebellar long-term depression. *Neuron* 49:845–860
- Terashima A, Pelkey KA, Rah JC, Suh YH, Roche KW, Collingridge GL, McBain CJ, Isaac JT (2008) An essential role for PICK1 in NMDA receptor-dependent bidirectional synaptic plasticity. *Neuron* 57:872–882
- Wang YN, Zhou L, Li YH, Wang Z, Li YC, Zhang YW, Wang Y, Liu G, Shen Y (2015) Protein interacting with C-Kinase 1 deficiency impairs glutathione synthesis and increases oxidative stress via reduction of surface excitatory amino acid carrier 1. *J Neurosci* 35:6429–6443
- Liu B, Wang K, Gao HM, Mandavilli B, Wang JY, Hong JS (2001) Molecular consequences of activated microglia in the brain: overactivation induces apoptosis. *J Neurochem* 77:182–189
- Anggono V, Clem RL, Huganir RL (2011) PICK1 loss of function occludes homeostatic synaptic scaling. *J Neurosci* 31:2188–2196
- Zhu J, Shao CY, Yang W, Zhang XM, Wu ZY, Zhou L, Wang XX, Li YH, Xia J, Luo JH, Shen Y (2012) Chronic zinc exposure decreases the surface expression of NR2A-containing NMDA receptors in cultured hippocampal neurons. *PLoS One* 7:e46012
- Xie YJ, Zhou L, Jiang N, Zhang N, Zou N, Zhou L, Wang Y, Cowell JK, Shen Y (2015) Essential roles of leucine-rich glioma inactivated 1 in the development of embryonic and postnatal cerebellum. *Sci Rep* 5:7827
- Nguyen TT, Kim YM, Kim TD, Le OT, Kim JJ, Kang HC, Hasegawa H, Kanaho Y, Jou I, Lee SY (2013) Phosphatidylinositol 4-phosphate 5-kinase  $\alpha$  facilitates Toll-like receptor 4-mediated microglial inflammation through regulation of the Toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP) location. *J Biol Chem* 288:5645–5659
- Neniskyte U, Neher JJ, Brown GC (2011) Neuronal death induced by nanomolar amyloid  $\beta$  is mediated by primary phagocytosis of neurons by microglia. *J Biol Chem* 286:39904–39913
- Wang DJ, Su LD, Wang YN, Yang D, Sun CL, Zhou L, Wang XX, Shen Y (2014) Long-term potentiation at cerebellar parallel fiber-Purkinje cell synapses requires pre- and postsynaptic signaling cascades. *J Neurosci* 34:2355–2364
- Wu ZY, Zhu LJ, Zou N, Bombek LK, Shao CY, Wang N, Wang XX, Liang L, Xia J, Rupnik M, Shen Y (2012) AMPA receptors regulate exocytosis and insulin release in pancreatic  $\beta$  cells. *Traffic* 13:1124–1139
- Mosbacher J, Maier R, Fakler B, Glatz A, Crespo J, Bilbe G (1998) P2Y receptor subtypes differentially couple to inwardly-rectifying potassium channels. *FEBS Lett* 436:104–110
- Liu GD, Ding JQ, Xiao Q, Chen SD (2009) P2Y<sub>6</sub> receptor and immunoinflammation. *Neurosci Bull* 25:161–164
- Deken SL, Beckman ML, Quick MW (2001) PICKing on transporters. *Trends Neurosci* 24:623–625
- Cao M, Mao Z, Kam C, Xiao N, Cao X, Shen C, Cheng KKY, Xu A, Lee KM, Jiang L, Xia J (2013) PICK1 and ICA69 control insulin granule trafficking and their deficiencies lead to impaired glucose tolerance. *PLoS Biol* 11:e1001541