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ORIGINAL ARTICLE

NPP1 is responsible for potent extracellular ATP hydrolysis as NTPDase1 in primary cultured murine microglia

Hye Min Lim[1] · Woon Heo[1] · Jung Woo Han[1] · Min Goo Lee[1] · Joo Young Kim[1]

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Abstract

The movement of microglia is regulated mainly by P1 and P2 purinergic receptors, which are activated by various nucleotides and their metabolites. Recently, such purinergic signalling has been spotlighted because of potential roles in the pathophysiologies of neurodegenerative and neuropsychiatric disorders. To understand the characteristics of microglia in relation of P1 and P2 signalling, we investigated the ectoenzymes expressed in microglia. At first, we profiled the expression of all known ectoenzymes in cultured microglia. We found that, like NTPDase1 (ectonucleoside triphosphate diphosphohydrolase 1, CD39), NPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1, PC-1) is also highly expressed in primary cultured murine microglia. Knockdown of NPP1 significantly reduced ATP hydrolysis and P_i production in cultured microglia. In addition, the knockdown of NPP1 enhanced basal nucleotide-stimulating responses of cultured microglia, such as phagocytosis and cell migration, and these results were very similar to NTPDase1 knockdown results. Moreover, inhibition of the adenosine receptors by caffeine treatment reduced phagocytosis of NPP1 knock downed-cultured microglia. In conclusion, we suggest that these potent ectoenzymes of primary cultured murine microglia, NPP1 together with CD73 (ecto-5′-nucleotidase) maintain the adenosine levels for triggering nucleotide-stimulating responses.

Keywords Microglia · Ectoenzyme · NTPDase1 · NPP1 · Migration · Phagocytosis

Introduction

Much of what is known about ATP primarily concerns its function as an intracellular energy unit, and the mechanisms by which this nucleotide and its various metabolites act as signalling molecules remain largely unexplored [1]. An understanding of ATP and its metabolites in purinergic signalling is important because it explains many aspects of the pathophysiologies of neurodegenerative and neuropsychiatric disorders [2–4].

ATP secreted from live cells or released from ruptured dead cells is metabolised by four major groups of ectonucleotidases, including the ectonucleoside triphosphate diphosphohydrolases (NTPDases), ecto-5′-nucleotidase (CD73), ectonucleotide pyrophosphatase/phosphodiesterases (NPPs) and alkaline phosphatases [5–8]. NTPDase1 (also known as CD39), is the most researched ectoenzyme, with functions in microglia [5, 9, 10]. In the case of NPP1 (or PC1), it is a known regulator of blood vessel calcification and PP_i concentration [11], and its mutation causes the "tiptoe walking" phenotype in mice [11–13], although NPP1 expression in microglia was firstly reported by Zhang et al. [14], but its function in microglia was not studied yet.

Microglia are resident macrophages and constitute a total of 5–10% of cells in the central nervous system (CNS) [15–17]. Recent evidence has shown that the microglia have diverse functions in the development and plasticity of CNS [18–20]. Interestingly, extracellular ATP can act as a warning signalling molecule via microglial activation [21]. The migration and phagocytic activity of microglia are controlled by signalling through purinergic receptors [17, 22–24], which are among the most abundant signalling receptors in living organisms [6]. Extracellular ATP is degraded by ectoenzymes to ADP, AMP and adenosine, which bind various types of ATP and adenosine (P2 and P1, respectively) receptors to

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✉ Joo Young Kim
jooyoungkim@yuhs.ac

[1] Department of Pharmacology and Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea

control a wide array of cellular responses [2, 21]. Ionotropic P2X ATP receptors, of which there are seven ($P2X_1$–$P2X_7$), were identified according to their mechanisms of action, pharmacology and molecular cloning [2]. Metabotropic P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$–$P2Y_{14}$) are coupled to heteromeric G-proteins to induce intracellular second-messenger signalling [6]. Similarly, the P1 receptor subtypes A1 and A3 are G_i coupled, whereas A2a and A2b are G_s coupled receptors [2, 25]. In microglia, it has been suggested that ATP induces a "modal shift" of activation, transitioning from P2 activation to P1 activation as ATP is metabolised to adenosine in the extracellular space [5].

We accidentally discovered that cultured microglia have an outstanding ATP degradation capacity using a Glu-titre system, which measures ATP concentrations for analyses of cell survival. We used this system to compare the capacities of cultured astrocytes and neurons to metabolise ATP and found that cultured microglia were superior in this regard. The previous reports that brain slices of NTPDase1-knockout mice that show almost no inorganic phosphate (P_i) production suggested that NTPDase1 works as a main ectoenzyme hydrolysing ATP in microglia [10, 26]. However, we hypothesised that another enzyme may contribute to cultured microglial ATP degradation on the bases that (i) the profile of ectoenzymes expression in cultured microglia is not known and (ii) there are no small interfering RNA (siRNA) screening studies to confirm that NTPDase1 is the only ectoenzyme in microglia.

In this study, we investigated the expression of all known ectoenzymes in cultured microglia and found that, in addition to NTPDase1, NPP1 is also highly expressed. Moreover, knockdown of NPP1 significantly inhibited P_i production after addition of ATP. Like NTPDase1, NPP1 is involved in the adenosine-producing process together with CD73 in cultured microglia in the resting state to maintain a threshold sufficient for triggering a nucleotide-stimulating purinergic response.

Materials and methods

Cell culture

Female C57BL/6 mice (weight, 20–25 g, 14~17 day of gestation) were purchased from Orient (Seongnam, South Korea). All experimental protocols in this study were approved by the Institutional Animal Research Ethics Committee at the Yonsei Medical Center (IACUC Approval No. 2017-0041-1). Primary cultures of microglia were prepared from the brains of embryonic day 17 mice. Briefly, cells were chemically and mechanically dissociated and seeded in DMEM/F12 medium (11320-033; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) [27]. After 3–4 weeks, microglia were isolated from primary mixed cells via mild trypsinisation [28] and shaking [29]. The upper cell layer of mixed cells was removed in one piece after incubation for 20 min at 37 °C in a trypsin 0.25% solution (SH30042.01; Hyclone, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) diluted 1:4 in DMEM/F12 medium containing 10% foetal bovine serum (26140-079; Gibco) and 1% penicillin-streptomycin (15140-122; Gibco). The remaining cultured microglia attached to the surface of the T75 flask were isolated by shaking at 120 rpm (SLOS-20; SeouLin Bioscience, Seoul, Korea) at 37 °C for 1 h. The supernatants were strained through a 40-μm cell strainer (352,340; BD Falcon, San Jose, CA, USA) and centrifuged at 4000 rpm at room temperature for 5 min. Glial cells were grown to a high density in T75 flasks.

To isolate the astrocyte, the mixed cell culture mentioned above was maintained in astrocyte-conditioned medium and microglia were depleted by adding 50 mM L-leucine-methyl ester (Sigma-Aldrich, St. Louis, MO, USA) for 4 h.

To culture cortical neurons, cells were prepared from embryonic day 14–15 mice (C57BL/6 strain) as described previously [19] with some modifications. The cerebral cortex was dissociated in an EDTA solution (Versene) and plated on laminin-coated (10 μg/mL in serum-free DMEM) plates. Cells were then incubated in DMEM supplemented with heat-inactivated horse serum (5%), foetal bovine serum (5%), 20 mM glucose, 38 mM sodium bicarbonate, and 2 mM L-glutamine. To inhibit the growth of non-neuronal cells, 7.5 μM Ara-C (cytosine-D-arabinoside; Sigma-Aldrich) was added to the medium 72 h after plating. Cortical neuronal cells were used at 9 days after primary culture.

The purities of the cell cultures were verified by immunostaining with anti-glial fibrillary acidic protein (GFAP) (clone GA5, MAB360; Millipore, Billerica, MA, USA), anti-Iba1 (019-19,741; Wako, Japan) and anti-microtubule-associated protein 2 (MAP2) (AB5622; Millipore) antibodies for glia, microglia and neurons, respectively. The numbers of cells were counted with a haemocytometer (Paul Marienfeld, Lauda-Königshofen, Germany).

RT-PCR and qPCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Thermo Fisher Scientific), and cDNA was synthesised using an AccuScript high-fidelity first-strand cDNA synthesis kit (200436; Agilent Technologies, Santa Clara, CA, USA). RT-PCR was performed with 100 ng of cDNA using Solg 2× *Taq* PCR Smart mix (STD02-M10h; SolGent Co., Daejeon, Korea). The thermocycling conditions for RT-PCR were an initial denaturation at 95 °C for 5 min followed by 31 cycles of 95 °C for 30 s, 58 °C for 40 s and 72 °C for 40 s, and a final extension at 72 °C for 7 min. The primer sets are provided in Supplementary Table 1. RT-PCR results were quantified by calculating the band intensity using Multi Gauge software (Fuji Film).

The expressions of NTPDase1, NPP1 and NPP2 (negative control) were analysed by quantitative real-time PCR (qPCR). qPCR reactions were performed in triplicates using TOP real qPCR 2× PreMix (SYBR green with high ROX, RT501;

Enzynomics, Daejeon, Korea). Each qPCR reaction contained 25 ng of cDNA. The 18s rRNA primer set was used as a loading control.

RNAi screening

Specific SMARTpool siRNAs were purchased from Dharmacon (Lafayette, CO, USA) as listed in Supplementary Table 2. For siRNA transfections, cultured microglia were seeded into 48-well plates at 5×10^4 cells/well and incubated overnight at 37 °C with a 5% CO_2 atmosphere. Then, 25 nM of each siRNA was transfected with Lipofectamine RNAi MAX transfection reagent (13778030; Invitrogen) in Opti-MEM (31985-070; Gibco) overnight [19]. Seventy-two hours after transfection, P_i production or ATP hydrolysis activity was measured.

Measurement of ATP concentrations

To measure ATP and metabolite levels, we used a luciferase-dependent ATP determination kit (A22066; Molecular Probes, Thermo Fisher Scientific) and a CellTiter-Glo luminescent cell viability assay (G7570; Promega, Madison, USA) according to the manufacturers' protocols. For these assays, the culture media for astrocytes, neurons and microglia (3.5×10^5 cells/well in 24-well plates) were changed for 120, 60, 30, 15 or 0 min with media with or without 50, 300 or 1000 μM ATP in regular solution (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES [pH 7.4, adjusted with NaOH] and 2 mM $CaCl_2$). At the endpoint of the assay, 500 μL of solution from each well was placed in 1.5-mL microcentrifuge tubes and mixed with 50 μL of the assay reagent. Next, 100 μL of these solutions was placed in a white 96-well plate for reading on an LB 960 microplate luminometer Centro XS^3 (Berthold Technologies GmbH & Co. KG, Germany).

P_i measurement

The production of inorganic phosphate (P_i) from siRNA-transfected cells was detected using a malachite green phosphatase assay kit (K-1500; Echelon Biosciences, Inc., UT, USA). The culture medium of each well of siRNA-transfected microglia was changed after 72 h with 200 μL of regular solution containing 50 μL of ATP stock (final concentration; 50 μM ATP). After 10 min, the supernatants were collected, and 20 μL was mixed with the malachite green (in regular solution, up to 100 μL) according to the manufacturer's protocol. The mixtures were then incubated in a transparent 96-well plate at room temperature for 10 min, and the absorbance at 610 nm was read using a VersaMax spectrophotometer plate reader (Molecular Devices, Sunnyvale, CA, USA). For the assessment of ATP hydrolysis by enzymes released into the medium, the cells were incubated for 10 min with 300 μM ATP, but the only supernatants were further incubated for 50 min prior to mixing with malachite green. For the quantitation of total protein from cells, 100 μL/well of 0.02% SDS was treated and then BCA assay was used.

Phagocytosis assay

Cultured microglia were seeded in 12-well plates at 3×10^5 cells/well and transfected as described in "RNAi screening" section. Transfected microglia were treated with FITC-labelled *E. coli* opsonised beads (conjugated with Alexa Fluor 488, E-13231; Molecular probes) for 2 h at 37 °C, 5% CO_2 incubator with or without 100 μM uridine 5′-diphosphate (UDP). After washing with phosphate-buffered saline, cells were treated with 0.4% trypan blue (15250-061; Gibco) at room temperature for 2 min to quench the particle fluorescence. Cells were fixed with a 4% formaldehyde solution and nuclei were stained with 5 μg/mL DAPI. Cells were visualised on an inverted fluorescence microscope (IX73-F22PH; Olympus), and fluorescence intensity was quantified with MetaMorph software (Molecular Devices). For flow cytometry, cells were treated with FITC-labelled *E. coli* opsonised beads in the presence or absence of UDP as described above and then treated with 0.05% trypsin-EDTA (25300-062; Gibco) and harvested by centrifugation at 2000 rpm at 4 °C for 5 min. The cell pellets were resuspended in 500 μL ice-cold serum-free medium and analysed using a BD FACSVerse (BD Biosciences, Franklin Lakes, NJ, USA) with FlowJo software (Treestar, Ashland, OR, USA).

Migration assay

Analyses of cultured microglial migration were performed by quantifying the numbers of cells migrating into a scratched area as previously described [24, 30]. All of scramble siRNA control and siRNA-transfected microglia were scratched with a scraper, and the scratched region of the plate was imaged by light microscopy. After 7 h, the same regions were imaged. Cell counting was performed using MetaMorph software (Molecular Devices).

Statistical analyses

Data are presented as the means ± standard errors of the means. Statistical analyses were performed with Student's *t* tests or with one-way or two-way analyses of variance (ANOVAs) followed by Tukey's multiple comparisons using the GraphPad Prism software package (version 5.0), as appropriate. A P value of < 0.05 was considered statistically significant.

Results

Extracellular ATP hydrolysis by cultured microglia

To compare ATP hydrolysis between cultured microglia and astrocytes, we measured ATP concentrations using a luciferase assay (Fig. 1a). Cultured microglia treated with 50 μM ATP showed rapid ATP hydrolysis, with all ATP degraded within 1 h. In contrast, astrocytes showed almost no ATP hydrolysis until 2 h. Alternatively, P_i production from ATP hydrolysis was also compared in cultured microglia and astrocytes (Fig. 1b). Cells were treated with 50, 300 and 1000 μM ATP, and time-dependent P_i production was compared using a malachite green colorimetric assay, which is more stable for long-term measurements than the luciferase assay. Cultured microglia treated with 50 μM ATP showed almost maximal P_i production within 20 min and maximal production at 60 min when treated with 300 μM ATP. Addition of 1000 μM ATP showed the similar P_i production of 300 μM ATP, which implied that 300 μM ATP is enough to see the maximum function of the enzymes in our experiment condition. By contrast, astrocytes showed minimal P_i production upon treatment with any of the three ATP concentrations.

We then assessed ATP hydrolysis in activated cultured microglia treated with 100 ng/mL lipopolysaccharides (LPS). Interestingly, LPS did not significantly enhance ATP hydrolysis by microglia (Fig. 1c). These results demonstrate that cultured microglia rapidly and proficiently hydrolyse ATP compared with that of cultured astrocytes. Furthermore, this high level of activity is not enhanced by microglial activation.

High expression of NTPDase1 and NPP1 mRNA in cultured microglia

To determine which enzyme is driving the potent extracellular ATP hydrolysis by cultured microglia, we performed RT-PCR for four groups of ectoenzymes in whole brain and cultured microglial samples: group 1, NTPDase1–NTPDase8; group 2, NPP1, NPP2 and NPP4–NPP6; group 3, ecto-5′-nucleotidase (Nt5e/CD73); group 4, AKP3, AKP5 and AKP6 (Fig. 2a and Supplementary Figs. 1 and 2). Notably, the expression levels of NTPDase1 and NPP1 transcripts were enriched in cultured microglia samples nearly sixfold and ~ threefold, respectively, compared with the expression levels in the whole brain samples. On the basis of the results of this RT-PCR analysis, we considered expression levels of at least 50% of that of whole brain as indicative of specific expression in cultured microglia, namely, NTPDase1, NTPDase4, NTPDase5, NTPDase7, NPP1, NPP4, CD73, AKP3, AKP5 and AKP6 (Fig. 2a, b). We further confirmed by qPCR that cultured microglia has high expression of NTPDase1 and NPP1 by comparing their expressions in astrocytes and neurons (Fig. 2c, d).

siRNA screening of cultured microglial ectoenzymes

Using the above-defined ectoenzyme expression profile in cultured microglia, we next used siRNAs to knockdown each ectoenzyme in turn to discover which is responsible for driving the ATP hydrolysis activity. siRNA for NPP2 was included as a negative control, as it is not expressed in cultured microglia. After transfections with each siRNA construct, ATP hydrolysis activities were assayed via P_i production using a malachite green assay. However, cells expressing siEntpd5 and siEnpp4 exhibited 40 and 75% cell death, respectively, and so these were not included in subsequent analyses. Cells with at least 70% survival 72 h after siRNA transfection were treated with 50 μM ATP for 10 min, and P_i production was measured. Figure 3a shows the results from three independent experiments. Most notably, siRNA-induced knockdown of NTPDase1, NTPDase4 and NPP1 significantly reduced P_i production relative to the scramble siRNA control. As NPP1, AKP3, AKP5 and AKP6 are enzymes released into

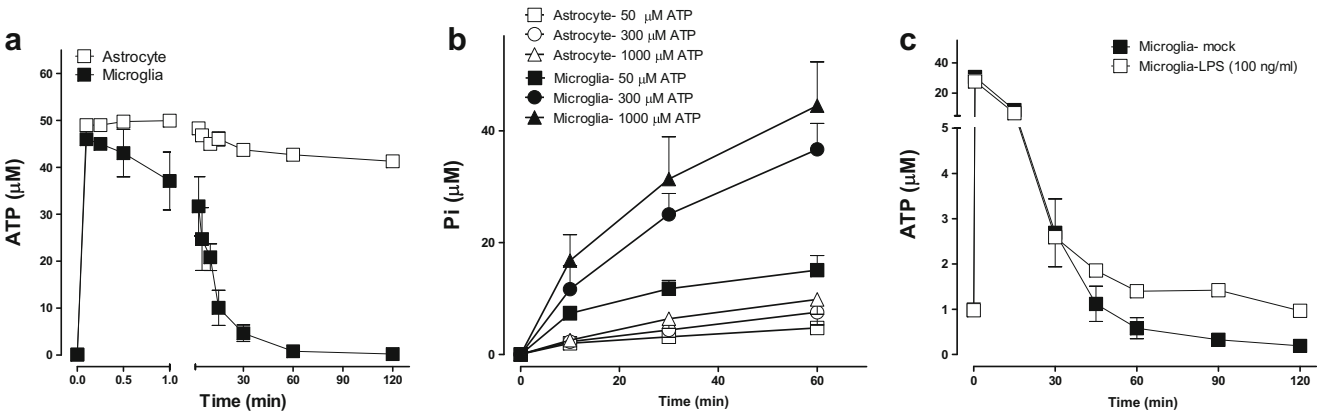

Fig. 1 ATP hydrolysis by primary cultured murine microglia. **a** ATP hydrolysis by microglia and astrocytes was measured by a luciferase assay. In the assay, 50 μM ATP was initially added to the medium and the time-dependent degradation of ATP was measured. **b** Time- and dose-dependent production of P_i using a malachite green-based colorimetric assay in microglia and astrocytes treated with 50, 300 and 1000 μM ATP. **c** ATP was measured in resting microglia and in fully activated microglia treated with 100 ng/mL LPS for 24 h

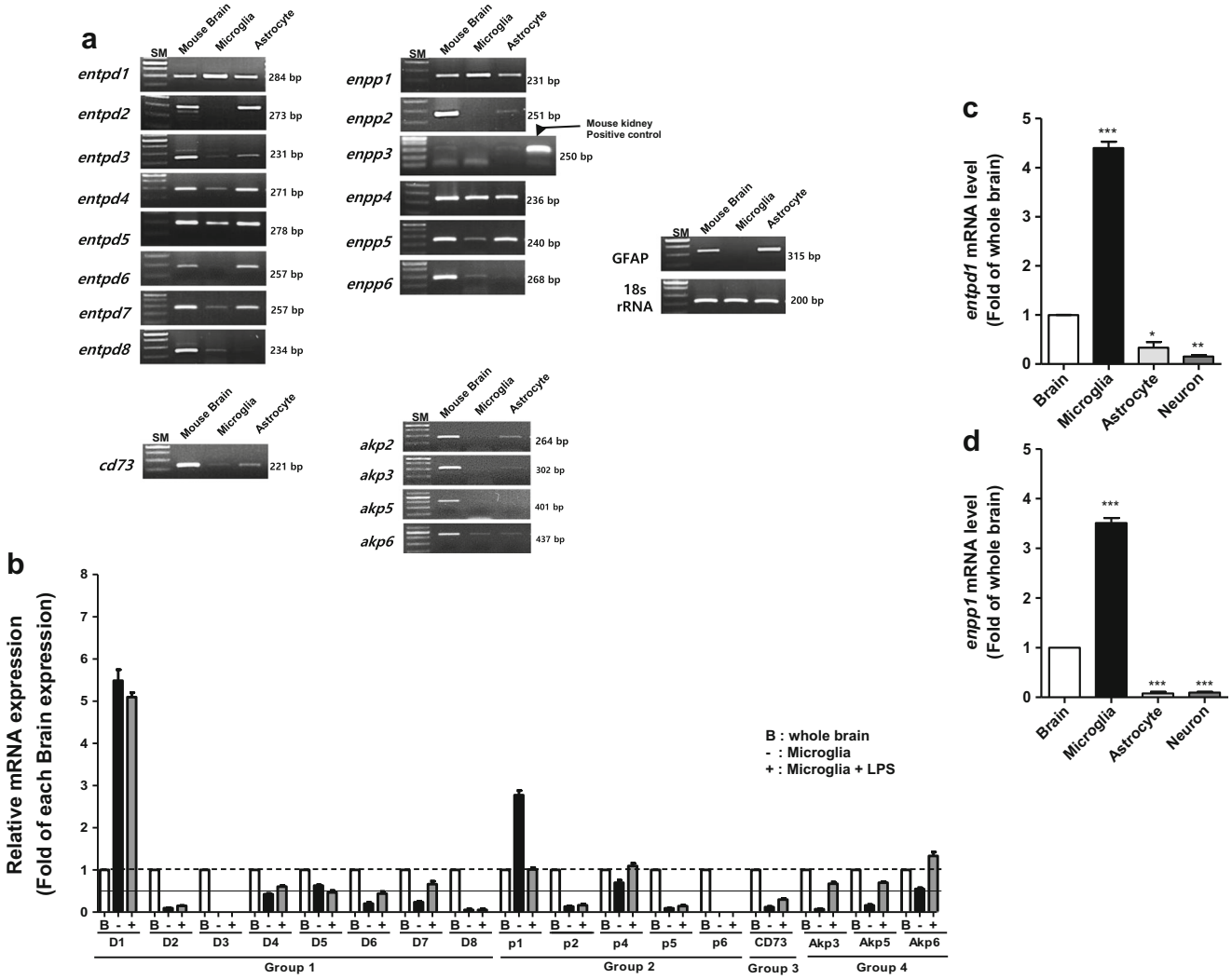

Fig. 2 Expression of ectoenzymes in cultured microglia. **a** Relative mRNA expression levels of various ectoenzymes in whole brain and in resting and activated microglia were assessed by RT-PCR anaysis. Levels are relative to those from whole brain samples (dotted line). The thin solid line denotes the 50% expression cutoff used to define microglial expression. Group 1, D1–D8 for NTPDase1–NTPDase8; group 2, p1, p2 and p4–6 for NPP1, NPP 2 and NPP 4–NPP 6, respectively; group 3, CD73 for ecto-5′-nucleotidase; group 4, AKP3, AKP5 and AKP6 for AKP 3, AKP5 and AKP6, respectively. The relative enrichment of NTPDase1 (**b**) and NPP1 (**c**) was specifically from cultured microglia, as significantly decreased levels were observed in cultured astrocytes and neurons in qPCR analyses. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

the extracellular space [6, 31], we also tested the ATP-hydrolysing activities of supernatants of cells transfected with the corresponding siRNAs. 300 μM ATP was added to each cells for 10 min, and then the supernatants were collected and incubated for further 50 min to analyse hydrolysis of ATP via released enzymes. Interestingly, only the supernatants from cells transfected with siNPP1 showed significantly reduced P_i production relative to the scramble siRNA control, while siAKP5 and siCD73 showed more enhanced Pi production (Fig. 3b). Additionally, we measured P_i production in cells receiving double transfections of siNTPDase1 and siNPP1; however, the cell survival was significantly reduced, suggesting that activity of at least one of these is required to maintain the viability of cells (Supplementary Fig. 3). Nevertheless, the data suggest that NTPDase1 and NPP1 are the primary contributors to cultured microglial ATP hydrolysis and that NPP1 is the chief secreted enzyme from cultured microglia for ATP hydrolysis.

siNPP1 increases basal phagocytosis and migration of cultured microglia

The physiological roles of NPP1 were investigated by phagocytosis and cell migration assays in cultured microglia transfect with siNPP1. To assess nucleotide-stimulating purinergic responses, cultured microglia were first treated with 100 μM UDP, which enhances phagocytosis via $P2Y_6$ signalling, and the phagocytosis of FITC-labelled *E. coli* opsonised beads was assessed via flow cytometry. UDP treatment time-dependently increased

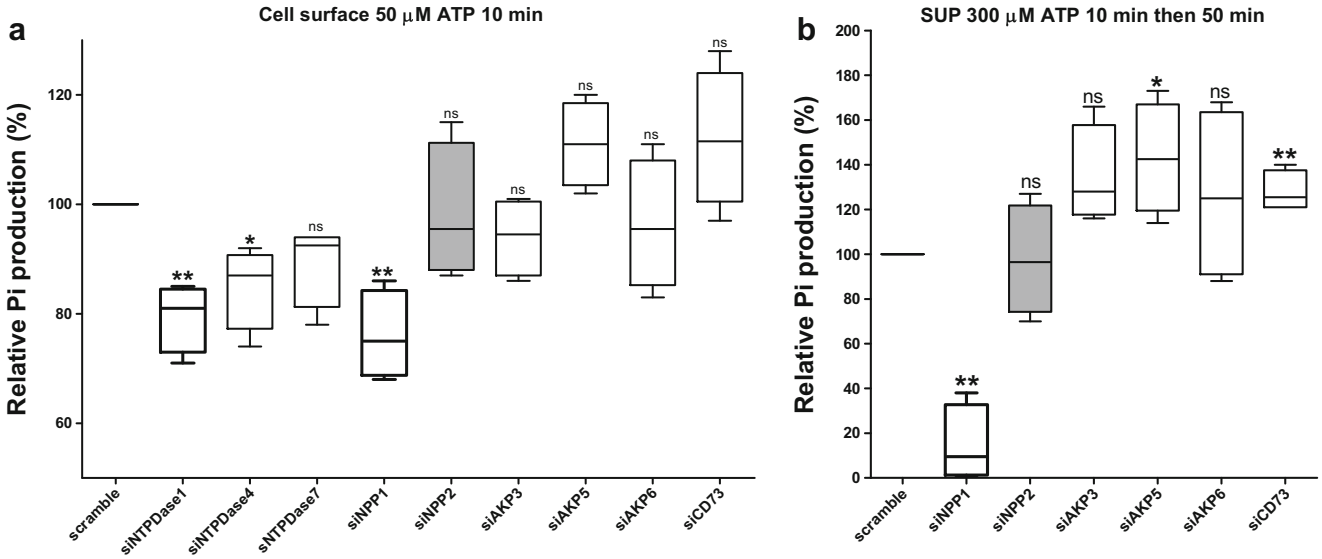

Fig. 3 siRNA screening for ectoenzymes with ATP hydrolysis activity in cultured microglia. **a** Microglia were transfected with individual siRNAs targeting ectoenzymes identified by RT-PCR, and the relative production of P_i was assessed after addition of 50 μM ATP for 10 min. **b** P_i production by secreted enzymes was assessed in superantants collected and further incubated 50 min after addition of 300 μM ATP for 10 min. Scramble siRNA and siNPP2 were used as negative controls, and siNPP2 were indicated by filled grey boxes. $*p < 0.05$; $**p < 0.01$. ns not significant

the phagocytosis of fluorescent beads as evidenced by increased fluorescence (Fig. 4a). Interestingly, siNPP1-transfected microglia showed basal phagocytic activity that was significantly higher than that of scramble siRNA controls (Fig. 4b). The enhanced phagocytosis of siNPP1-transfected microglia was also observed in phagocytosis analysis of cultured microglia on the coverslip, and siNTPDase1-transfected microglia also showed the similar result of siNPP1 (Supplementary Fig. 4). In a cell migration assay, scramble siRNA-transfected microglia showed increased migration into a scratched area upon ATP stimulation. Similar to the observations regarding phagocytosis, microglia transfected with siNPP1 showed greater migration compared with scramble siRNA controls, and this activity was inhibited by ATP addition (Fig. 4c, d). Together, these data indicate that knockdown of NPP1 enhances nucleotide-stimulating purinergic responses in resting microglia.

Inhibition of adenosine signalling reverses enhanced phagocytosis in cultured microglia with NPP1 knockdown

To assess the role of adenosine signalling in siNPP1-transfected microglia, cells were treated for 30 min with 50 μM caffeine and an inhibitor of adenosine receptors. Caffeine treatment of scramble siRNA control cells significantly increased the phagocytosis of FITC-labelled beads to an extent similar to that with UDP treatment. By contrast, phagocytic activity of siNPP1-transfected microglia, which show enhanced activity compared with controls and are not affected by UDP, was significantly suppressed by caffeine (Fig. 5a, b). The enhancement of phagocytosis of siNPP1-transfected microglia at the resting state may result from reduced adenosine production due to the deficient extracellular ATP hydrolysis.

Discussion

In this study, we found that the primary cultured murine microglia predominantly expressed NTPDase1 and NPP1. In addition, we found that the inhibition of the NTPDase1 or NPP 1 enzyme activities by siRNA showed a high nucleotide stimulating activity in the basal condition. This tendency was especially prominent in the cell migration experiment. These data indicated that the ectoenzymes supplied a certain level of adenosine via hydrolysing the secreted small amount of ATP from the cells. Our results confirmed previous findings by Bulavina et al. [10] and Braun et al. [9] demonstrating the importance of NTPDase1 in microglial purinergic responses and ATP hydrolysis, respectively, via experiments with NTPDase1-knockout mice.

In addition to termination of nucleoside triphosphate receptor activation and creation of agonist for nucleoside diphosphate-sensitive receptors, producing adenosine is also a critical role of microglial ectoenzymes. Adenosine generated by NPP1 and NTPDase1 seems to set the threshold for triggering purinergic responses via activating P1 receptors in the resting state. Indeed, RT-PCR

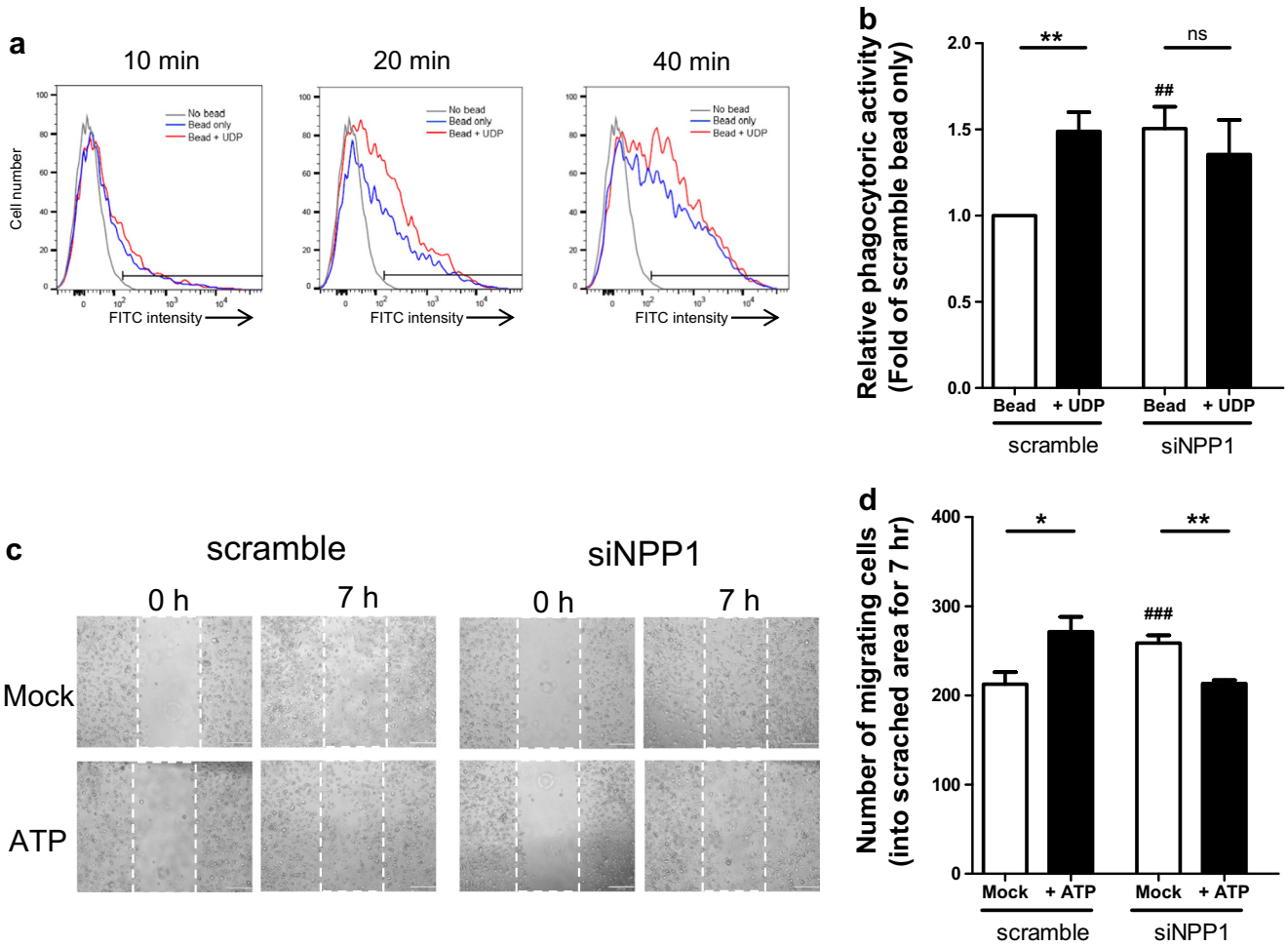

Fig. 4 NPP1 knockdown increases phagocytosis and migration of resting microglia. **a** Representative results from fluorescence-activated cell sorting analysis of phagocytic uptake of FITC-labelled beads by microglia with or without 100 μM UDP after 10, 20 and 40 min. **b** Quantification of regions of fluorescence from fluorescence-activated cell sorting analysis summarised from geometric means and shown as fold difference relative to the bead-only condition (Bead) of the scramble siRNA control. **c** Representative images showing the migration of microglia into a scratched region (between white dashed lines) immediately (0 h) and 7 h after scratching in the absence (mock) or presence of 50 μM ATP. **d** The numbers of control (scramble siRNA) or siNPP1-transfected microglia that migrated were counted from three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; ns not significant; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. scramble siRNA mock

analyses demonstrated that microglia express both G_i-coupled (A1 and A3) and G_s-coupled (A2a and A2b) P1 receptors, and their expression levels were not changed after NTPDase1 or NPP1 knockdown (Supplementary Fig. 5). Thus, the amount of adenosine produced by ectoenzymes rather than adenosine receptor expression is the factor determining the cellular responses. In addition, microglia also express P2 receptors which activate chemotaxis and phagocytosis [17, 25] and are activated by ATP secreted from damaged or dying cells [22]. Further studies manipulating the P1 and P2 receptors will uncover the detailed molecular mechanisms of enhanced nucleotide-stimulating purinergic responses after the knockdown of NTPDase1 or NPP1 in cultured microglia.

The pre-treatment of scramble siRNA transfected microglial with caffeine increased phagocytic activity as UDP treatment (Fig. 5). However, in the case of siNPP1-transfected microglia, phagocytosis was dramatically attenuated by caffeine which inhibits the adenosine receptor. These results suggested the following: (1) Phagocytosis is normally suppressed by adenosine, which is continuously produced by NPP1; (2) however, when phagocytosis is suppressed by adenosine in this way, inhibition of adenosine signalling by caffeine seems to rather activate the phagocytosis activity; and (3) enhanced phagocytosis following Enpp1 knockdown at the resting state is due to the depletion of adenosine, which is no longer increased by further UDP treatment. We therefore hypothesise that producing adenosine by NPP1 might maintains the threshold for triggering nucleotide-stimulating purinergic responses. Very strangely, when treated with adenosine inhibitor, phagocytosis was

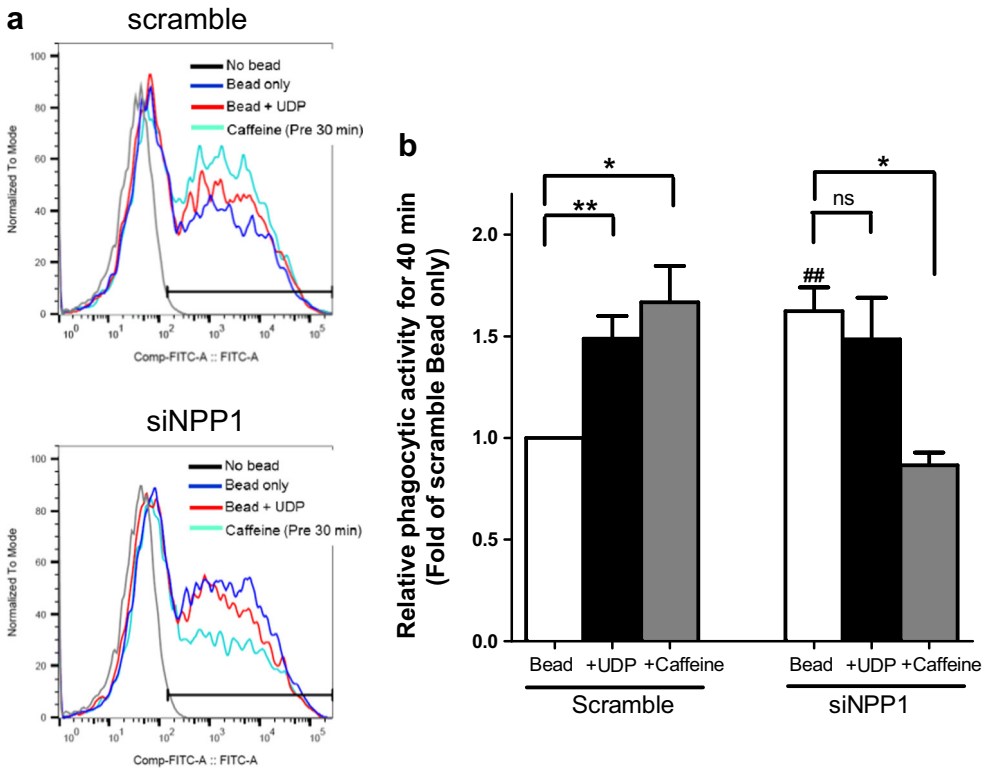

Fig. 5 Inhibition of adenosine receptors mimics the effects of UDP on cultured microglial phagocytosis. **a** Representative result from fluorescence-activated cell sorting analysis of phagocytic uptake of FITC-labelled beads by microglia 40 min after stimulation by 100 μM UDP or 50 μM caffeine (30 min pre-treatment before adding beads). **b** Quantification of regions of fluorescence from fluorescence-activated cell sorting analysis summarised from geometric means and shown as fold difference relative to the bead-only condition (mock) of the scramble siRNA control. $^{*}p < 0.05$; $^{**}p < 0.01$; ns not significant; $^{\#\#}p < 0.01$ vs. from scramble siRNA mock

dramatically reduced, which is difficult to explain at this time. Previous studies have reported that ATP sensitivity is increased by adenosine on the basis that ATP-induced current was maintained by co-treatment with adenosine [26, 32, 33]. These results also supported the notion that ectoenzymes such as NPP 1 provide adenosine under normal conditions.

In our experiments, siRNA knockdown of NPP4 or NTPDase5 caused severe cell death (approximately 70 or 40%, respectively), which disturbed the accurate measurement of ATP hydrolysis. Thus, these enzymes might not work as ectoenzymes in microglia but are critical factors for microglial survival. Further investigation of their role in microglia is needed to understand the exact function of each enzyme. In addition, inhibition of both NTPDase1 and NPP1 caused cell death of 50% of microglia (Supplementary Fig. 3). This might imply that the activity of at least one enzyme is required to maintain cell survival.

Recently, Matyash et al. reported that CD39/CD73 is mainly in charge as an adenosine-producing enzyme in microglia which controls the microglial process ramification in mouse brain [34]. In accordance, we suggested that in cultured microglia, NPP1 together with CD73 fulfils a similar role in adenosine production. In addition, NPP1 and CD73 are secreted proteins [5, 6]; these enzymes might remain stationary and exhibit functional activity when microglia is migrating. Considering the high mobility of the cell, the secreted protein is thought to confer properties related to migration.

Microglial ectoenzymes have not been studied extensively. This may be due in part to the complexity of purinergic signalling and the difficulty with analysing intracellular and secreted ATP and its metabolites. Nonetheless, our findings demonstrate the importance of ectoenzymes in microglia for maintaining basal cell responsiveness by producing adenosine. The production of adenosine by microglia may be to minimise irritation to nearby cells during migration. Considering the secreted characteristics of NPP1, it is possible that NPP1 is able to sustain the production of adenosine in situ by remaining in the pathway of microglia movement. Accordingly, we demonstrated that microglia have an extraordinarily powerful ATP hydrolysis via NTPDase1 and NPP1, which strongly support the modal shift phenomena in ATP-stimulated microglia.

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Author contribution JYK conceived of and designed the experiments. HML performed most of the experiments. HW and JWH helped to produce the primary culture of microglia. JYK and HML analysed the data. MGL participated in design the experiments and contributed reagents, materials and instruments. JYK wrote the manuscripts. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflicts of interest Hye Min Lim declares that she has no conflict of interest.

Woon Heo declares that he has no conflict of interest.

Jung Woo Han declares that he has no conflict of interest.

Min Goo Lee declares that he has no conflict of interest.

Joo Young Kim declares that he has no conflict of interest.

Ethical approval All experimental protocols in this study were approved by the Institutional Animal Research Ethics Committee at the Yonsei Medical Center (IACUC Approval No. 2017-0041-1).

Abbreviations CNS, Central nerve system; NPP, Ectonucleotide pyrophosphatase/phosphodiesterase; GFAP, Glial fibrillary acidic protein; LPS, Lipopolysaccharides; MAP2, Microtubule-associated protein 2; NTPDase, Ectonucleoside triphosphate diphosphohydrolase; UDP, Uridine 5′-diphosphate

References

1. Burnstock G (2012) Purinergic signalling: its unpopular beginning, its acceptance and its exciting future. BioEssays 34(3):218–225. https://doi.org/10.1002/bies.201100130
2. Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H (2009) Purinergic signalling in the nervous system: an overview. Trends Neurosci 32(1):19–29. https://doi.org/10.1016/j.tins.2008.10.001
3. Czirr E, Wyss-Coray T (2012) The immunology of neurodegeneration. J Clin Invest 122(4):1156–1163. https://doi.org/10.1172/jci58656
4. Block ML, Zecca L, Hong JS (2007) Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8(1):57–69. https://doi.org/10.1038/nrn2038
5. Robson SC, Sevigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2(2):409–430. https://doi.org/10.1007/s11302-006-9003-5
6. Zimmermann H, Zebisch M, Strater N (2012) Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal 8(3):437–502. https://doi.org/10.1007/s11302-012-9309-4
7. Joseph SM, Pifer MA, Przybylski RJ, Dubyak GR (2004) Methylene ATP analogs as modulators of extracellular ATP metabolism and accumulation. Br J Pharmacol 142(6):1002–1014. https://doi.org/10.1038/sj.bjp.0705865
8. Stefan C, Jansen S, Bollen M (2006) Modulation of purinergic signaling by NPP-type ectophosphodiesterases. Purinergic Signal 2(2):361–370. https://doi.org/10.1007/s11302-005-5303-4
9. Braun N, Sevigny J, Robson SC, Enjyoji K, Guckelberger O, Hammer K, Di Virgilio F, Zimmermann H (2000) Assignment of ecto-nucleoside triphosphate diphosphohydrolase-1/cd39 expression to microglia and vasculature of the brain. Eur J Neurosci 12(12):4357–4366
10. Bulavina L, Szulzewsky F, Rocha A, Krabbe G, Robson SC, Matyash V, Kettenmann H (2013) NTPDase1 activity attenuates microglial phagocytosis. Purinergic Signal 9(2):199–205. https://doi.org/10.1007/s11302-012-9339-y
11. Kato K, Nishimasu H, Okudaira S, Mihara E, Ishitani R, Takagi J, Aoki J, Nureki O (2012) Crystal structure of Enpp1, an extracellular glycoprotein involved in bone mineralization and insulin signaling. Proc Natl Acad Sci U S A 109(42):16876–16881. https://doi.org/10.1073/pnas.1208017109
12. Nam HK, Liu J, Li Y, Kragor A, Hatch NE (2011) Ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) protein regulates osteoblast differentiation. J Biol Chem 286(45):39059–39071. https://doi.org/10.1074/jbc.M111.221689
13. Okawa A, Nakamura I, Goto S, Moriya H, Nakamura Y, Ikegawa S (1998) Mutation in Npps in a mouse model of ossification of the posterior longitudinal ligament of the spine. Nat Genet 19(3):271–273. https://doi.org/10.1038/956
14. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, Phatnani HP, Guarnieri P, Caneda C, Ruderisch N, Deng S, Liddelow SA, Zhang C, Daneman R, Maniatis T, Barres BA, Wu JQ (2014) An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J Neurosci 34(36):11929–11947. https://doi.org/10.1523/jneurosci.1860-14.2014
15. Ransohoff RM, Cardona AE (2010) The myeloid cells of the central nervous system parenchyma. Nature 468(7321):253–262. https://doi.org/10.1038/nature09615
16. Prinz M, Priller J (2014) Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. Nat Rev Neurosci 15(5):300–312. https://doi.org/10.1038/nrn3722
17. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A (2011) Physiology of microglia. Physiol Rev 91(2):461–553. https://doi.org/10.1152/physrev.00011.2010
18. Salter MW, Beggs S (2014) Sublime microglia: expanding roles for the guardians of the CNS. Cell 158(1):15–24. https://doi.org/10.1016/j.cell.2014.06.008
19. Neher JJ, Neniskyte U, Zhao JW, Bal-Price A, Tolkovsky AM, Brown GC (2011) Inhibition of microglial phagocytosis is sufficient to prevent inflammatory neuronal death. J Immunol 186(8):4973–4983. https://doi.org/10.4049/jimmunol.1003600
20. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, Ragozzino D, Gross CT (2011) Synaptic pruning by microglia is necessary for normal brain development. Science 333(6048):1456–1458. https://doi.org/10.1126/science.1202529
21. Inoue K (2006) The function of microglia through purinergic receptors: neuropathic pain and cytokine release. Pharmacol Ther 109(1–2):210–226. https://doi.org/10.1016/j.pharmthera.2005.07.001
22. Domercq M, Vazquez-Villoldo N, Matute C (2013) Neurotransmitter signaling in the pathophysiology of microglia. Front Cell Neurosci 7:49. https://doi.org/10.3389/fncel.2013.00049
23. 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 P2Y6 receptors is a mediator of microglial phagocytosis. Nature 446(7139):1091–1095. https://doi.org/10.1038/nature05704
24. Ohsawa K, Irino Y, Nakamura Y, Akazawa C, Inoue K, Kohsaka S (2007) Involvement of P2X4 and P2Y12 receptors in ATP-induced microglial chemotaxis. Glia 55(6):604–616. https://doi.org/10.1002/glia.20489
25. Luongo L, Guida F, Imperatore R, Napolitano F, Gatta L, Cristino L, Giordano C, Siniscalco D, Di Marzo V, Bellini G, Petrelli R, Cappellacci L, Usiello A, de Novellis V, Rossi F, Maione S (2014) The A1 adenosine receptor as a new player in microglia physiology. Glia 62(1):122–132. https://doi.org/10.1002/glia.22592
26. Farber K, Markworth S, Pannasch U, Nolte C, Prinz V, Kronenberg G, Gertz K, Endres M, Bechmann I, Enjyoji K, Robson SC, Kettenmann H (2008) The ectonucleotidase cd39/ENTPDase1 modulates purinergic-mediated microglial migration. Glia 56(3):331–341. https://doi.org/10.1002/glia.20606
27. Heo DK, Lim HM, Nam JH, Lee MG, Kim JY (2015) Regulation of phagocytosis and cytokine secretion by store-operated calcium

entry in primary isolated murine microglia. Cell Signal 27(1):177–186. https://doi.org/10.1016/j.cellsig.2014.11.003
28. Saura J, Tusell JM, Serratosa J (2003) High-yield isolation of murine microglia by mild trypsinization. Glia 44(3):183–189. https://doi.org/10.1002/glia.10274
29. Tamashiro TT, Dalgard CL, Byrnes KR (2012) Primary microglia isolation from mixed glial cell cultures of neonatal rat brain tissue. J Vis Exp: JoVE 66:e3814. https://doi.org/10.3791/3814
30. de Jong EK, de Haas AH, Brouwer N, van Weering HR, Hensens M, Bechmann I, Pratley P, Wesseling E, Boddeke HW, Biber K (2008) Expression of CXCL4 in microglia in vitro and in vivo and its possible signaling through CXCR3. J Neurochem 105(5):1726–1736. https://doi.org/10.1111/j.1471-4159.2008.05267.x
31. Belli SI, van Driel IR, Goding JW (1993) Identification and characterization of a soluble form of the plasma cell membrane glycoprotein PC-1 (5′-nucleotide phosphodiesterase). Eur J Biochem 217(1):421–428
32. Inoue K, Watano T, Koizumi S, Nakazawa K, Burnstock G (1994) Dual modulation by adenosine of ATP-activated channels through GTP-binding proteins in rat pheochromocytoma PC12 cells. Eur J Pharmacol 268(2):223–229
33. Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG (2006) ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. Science 314(5806):1792–1795. https://doi.org/10.1126/science.1132559
34. Matyash M, Zabiegalov O, Wendt S, Matyash V, Kettenmann H (2017) The adenosine generating enzymes CD39/CD73 control microglial processes ramification in the mouse brain. PLoS One 12(4):e0175012. https://doi.org/10.1371/journal.pone.0175012