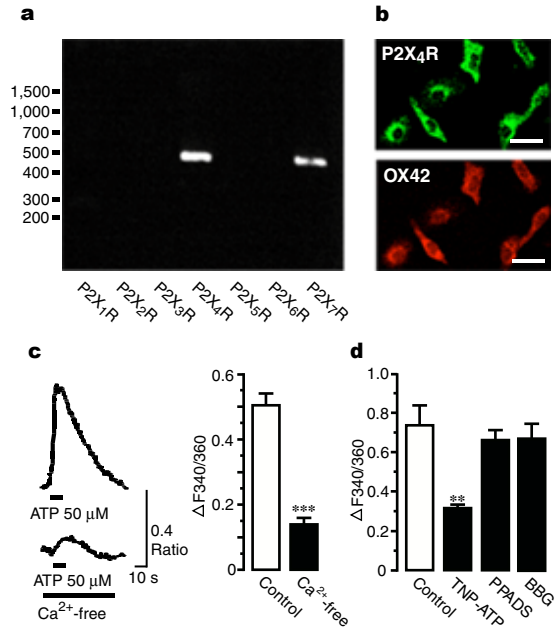

Supplementary Figure. Functional P2X4R is expressed in hyperactive microglia in primary culture. **a**, RT-PCR analysis of P2XR mRNAs from the extract of cultured microglia. **b**, Immunofluorescence of P2X4R protein (upper) and OX42 (lower) in microglia. **c**, **d**, Intracellular Ca^{2+} ($[Ca^{2+}]_i$) imaging analysis of individual microglia using the Ca^{2+}-sensitive fluorescent dye fura-2. The traces show ATP (50 μM, 10 s)-evoked a transient increase in the 340/360 emission ratio for fura-2 in microglia under the conditions with or without adding Ca^{2+} in the extracellular solution. The graphs show relative increase ratio (Δ340/F360; mean ± SEM) from the basal level before ATP application (***P<0.001, n=28 cells) (**c**). ATP (50 μM)-evoked a transient increase in the 340/360 emission ratio (Δ340/F360; mean ± SEM) is suppressed by pretreatment with 10 μM TNP-ATP (**P<0.01, n=14 cells), but not with 10 μM PPADS (n=26 cells) and with 100 nM brilliant blue G (BBG, n=21 cells), a selective antagonist for $P2X_7R$ (Jiang, L. H., Mackenzie, A. B., North, R. A. & Surprenant, A. Brilliant blue G selectively blocks ATP-gated rat $P2X_7$ receptors. Mol Pharmacol **58**, 82-88, 2000) (**d**).

Methods. Rat primary cultured microglia were prepared according to the method described previously (Nakajima, K. et al. Identification of elastase as a secretory protease from cultured rat microglia. J Neurochem **58**, 1401-1408, 1992). In brief, mixed glial culture was prepared from neonatal Wistar rats and maintained for 10-16 days in DMEM with 10% fetal bovine serum. Microglia were obtained as floating cells over the mixed glial culture. The floating cells were collected by a gentle shake and transferred to appropriate dishes or glasses, and then the microglia attached to them were used for RT-PCR, intracellular Ca^{2+} imaging, immunocytochemistry. RT-PCR was carried out as described previously (Shigemoto-Mogami, Y. et al. Mechanisms underlying extracellular ATP-evoked interleukin-6 release in mouse microglial cell line, MG-5. J Neurochem **78**, 1339-1349, 2001). Microglial cells (primary culture) were directly lysed with 0.5 ml of RNA STAT-60 (Tel-Test "B" Inc.) and total RNA was isolated. Reverse transcription was performed with 1 μg of total RNA using M-MLV reverse transcriptase. One μl of the RT product was added to the reaction mixture containing 1xPCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 units of Taq polymerase, and $P2X_1$-$P2X_7$ receptors specific primers;
$P2X_1R$ (296 bp): 5'-TCTTCTTCGTGAGGCTGAGA-3' and 5'-ACTGGTAGATGGGTTTGCAG-3',
$P2X_2R$ (358 bp): 5'-GAATCAGAGTGCAACCCCAA-3' and 5'-TCACAGGCCATCTACTTGAG-3',
$P2X_3R$ (462 bp): 5'-AAGTACCGCTGTGTGTCTGA-3' and 5'-ATCCAGCCGAGTGAAGGAAT-3',
$P2X_4R$ (515 bp): 5'-TCACCACGTCCTACCTCAAA-3' and 5'-CTGCTCGTAGTCTTCCACAT-3',
$P2X_5R$ (485 bp): 5'-ACACACACACTCCATCTCCT-3' and 5'-CTGCTTCACGTTCACAATGG-3',
$P2X_6R$ (411 bp): 5'-TAAGGAACTGGAGAAACGGC-3' and 5'-TAGGTGTTGTCCCAGGTATC-3',
$P2X_7R$ (497 bp): 5'-TAGTACACGGCATCTTCGAC-3' and 5'-CTGAACTGCCACCTCTGTAA-3'. After PCR amplification, the products were analysed by electrophoresis on agarose gel with ethidium bromide. Single-cell fluorescence monitoring of intracellular Ca^{2+}. $[Ca^{2+}]i$ in single microglial cells was monitored by using the Ca^{2+}-sensitive fluorescent dye fura-2. The microglia were incubated with 10 μM fura-2 acetoxymethylester for 45 min in DMEM. Then, the microglia were washed with balanced salt solution (BSS; composition in mM: NaCl 150, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.2, D-glucose 10 and HEPES 25; pH 7.4) and mounted on an inverted fluorescence microscope equipped with a Xenon-lamp and band-pass filters of 340 nm and 360 nm wavelength. The emission fluorescence was measured at 510 nm. Image data, recorded by a high-sensitivity silicon intensifier target camera, were processed by a Ca^{2+}-analyzing system (Furusawa Lab. Appliance. Co.). ATP (50 μM) was applied for 10 s. TNP-ATP (10 μM), PPADS (10 μM) and BBG (100 nM) were applied for 10 min before and during ATP application.