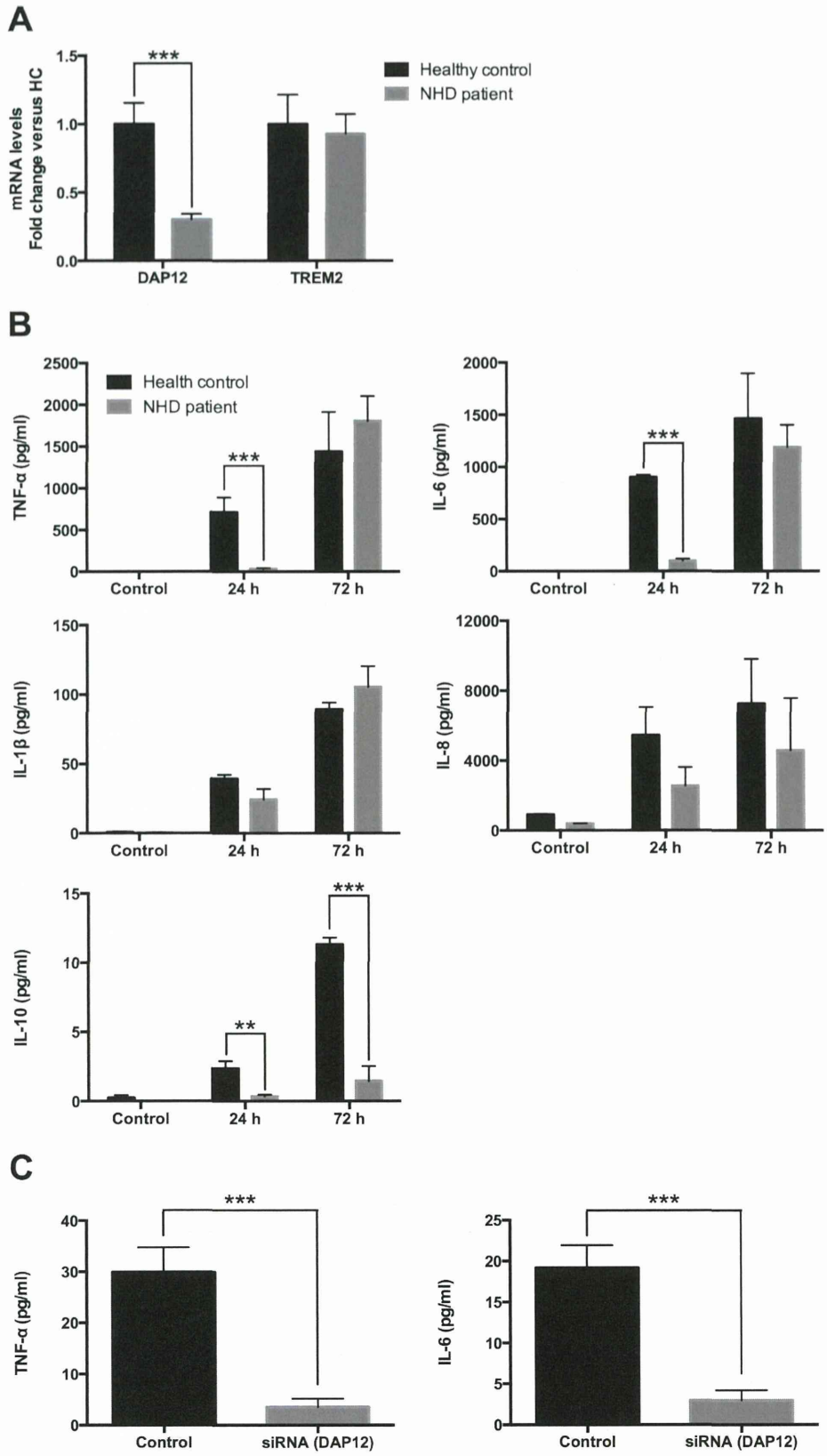


Figure 4 | Dynamic functional analysis of the iMG cells from a patient of NHD. (A) The iMG cells from the NHD patient showed significantly lower gene expression of DAP12 compared to those from the healthy control (n = 6). (B) Cytokine production from the iMG cells were compared between the NHD patient and the healthy control. The iMG cells from the NHD patient and the healthy control were incubated with latex beads for 24 or 72 hours, and culture supernatants were analyzed by CBA. In the iMG cells from the NHD patient, the production of pro-inflammatory cytokines (TNF-α and IL-6) was delayed, and that of anti-inflammatory cytokine (IL-10) was decreased (n = 4). (C) The effects of DAP12 silencing on the proinflammatory cytokine production. The iMG cells treated with siRNA were incubated with latex beads for 24 hours, and culture supernatants were analyzed by CBA In the iMG cells treated with siRNA, the production of pro-inflammatory cytokines was delayed (n = 8) $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars, SEM.

Methods

Subjects. The present study was conducted in accordance with the World Medical Association's Declaration of Helsinki and was approved by the Ethics Committee of the Graduate School of Medical Sciences, Kyushu University and Osaka University. We recruited a middle-aged female patient, who was diagnosed with Nasu-Hakola disease (141delG in DAP12 gene) in her thirties. Based on informed consents both from the patient and a family member, we took a blood sample. Healthy adult volunteers including an age-matched female were also recruited.

Induction of induced microglia-like (iMG) cells from human peripheral blood. Peripheral blood was collected using a heparinized tube from healthy adult volunteers and a patient of NHD. Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. PBMC were resuspended with RPMI-1640 (Nacalai Tesque, Kyoto, Japan), 10% heat-inactivated fetal bovine serum (FBS; Endotoxin = 0.692 EU/ml; Japan Bio Serum, Hiroshima, Japan) and 1% antibiotics/antimycotic (Invitrogen, Carlsbad, CA). PBMC were plated onto culture chambers at a density of 4×10^5 cells/ml and cultured overnight in standard culture conditions (37°C, 5% CO_2). After overnight incubation, culture supernatant and non-adherent cells were removed. The adherent cells (monocytes) were cultured with RPMI-1640 Glutamax (Invitrogen) supplemented with 1% antibiotics/antimycotic and a mixture of the following candidate cytokines; recombinant human GM-CSF (10 ng/ml; R&D Systems, Minneapolis, MN), recombinant human IL-34 (100 ng/ml; R&D Systems) and M-CSF (10 ng/ml; Peprotec, Rocky Hill, NJ) in order to develop iMG cells. We also developed induced macrophage from human monocytes; monocytes were cultured with RPMI-1640 Glutamax supplemented with 1% antibiotics/antimycotic and recombinant human GM-CSF (10 ng/ml). All cells were cultured in standard culture conditions for up to 14 days.

Cell morphology. Morphological changes of cytokines-treated cells were examined using phase-contrast microscopy (TS100-F; Nikon Instech, Tokyo, Japan). Images were taken with a DS-Vi1 digital camera (Nikon Instech) and a DS-L3 control unit (Nikon Instech).

Flow cytometry. Flow cytometry was performed using a FACS Aria (BD Biosciences, Bedford, MA) with FACS Diva software (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star, San Carios, CA). For iMG phenotyping, fluorochrome conjugated monoclonal antibodies specific for human CD11b (APC-Vio770; Miltenyi Biotec, Gladbach, Germany), CD14 (FITC; Sigma), CD45 (PE; Miltenyi Biotec) and CD200R (Alexa647; Serotec, Oxford, UK) were used. Induced macrophage and iMG cells were cultured in 6-well plates (Corning, NY) at a density of 4×10^5 cells/ml. Cells were harvested by non-enzymatic cell dissociation solution (Sigma) and cell lifter (Corning). The cells were washed with MACS buffer (Miltenyi Biotec) and incubated for 5 minutes at 4°C in FcR-blocking reagent (Miltenyi Biotec). Antibodies were incubated with cell suspension for 30 minutes at 4°C, washed with calcium-magnesium-free phosphate-buffered saline (PBS(-)), resuspended and fixed with 1% paraformaldehyde (Wako, Osaka, Japan) in PBS(-). The fluorescence intensity of the cells was measured.

Indirect immunofluorescence for flow cytometry was performed using the following antibodies: rabbit anti-CX3CR1 antibody (Immuno-Biological Laboratories, Gunma, Japan) and mouse anti-CCR2 antibody (R&D Systems). The monocytes and iMG cells were treated with the same process until the primary antibody staining. After primary staining, washed with MACS buffer and were stained with Alexa488- or Alexa546-conjugated secondary antibodies (Invitrogen). The ratio of CX3CR1 to CCR2 was calculated by the fluorescent intensity of each fluorochrome.

Immunocytochemistry. In immunocytochemistry, iMG cells and monocytes were cultured in 8-well chambers (LabTec chamber slide system; Nalge Nunc International, Rochester, NY) at a density of 4×10^5 cells/ml. These cells were fixed with 4% paraformaldehyde (Wako) for 20 minutes and then rinsed thrice with PBS(-) for 5 minutes. Indirect immunofluorescence was performed using the following antibodies: rabbit anti-CX3CR1 antibody (1 : 500 dilution; Immuno-Biological Laboratories, Gunma, Japan) and mouse anti-CCR2 antibody (1 : 500 dilution; R&D Systems). Cells were incubated in primary antibodies diluted in 0.1% Triton-X 100 in PBS containing 5% normal goat serum at 4°C overnight. After rinsing thrice with PBS(-) for 5 min, Alexa488- or Alexa546-conjugated secondary antibodies (Invitrogen) were used for detection. Fluorescent images were taken with a confocal laser scanning microscope (LSM-780; Carl Zeiss, Jena, Germany).

Quantitative real time-polymerase chain reaction (qRT-PCR). To assess the gene expression patterns in iMG cells after the treatment of IL-4, dexamethasone or during phagocytosis, we performed qRT-PCR using a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). IL-4 (40 ng/ml; Peprotec), dexamethasone (2 nM; Sigma) or latex beads-rabbit IgG-FITC solution (Cayman Chemical) was added to the iMG cells and incubated for 72 hours in standard culture conditions. After incubation, the iMG cells were washed and the total RNA was extracted using a High Pure RNA Isolation kit (Roche Diagnostics) according to the manufacturer's protocol, and subjected to cDNA synthesis using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). qRT-PCR for HLA-DR, CD45, TNF-α, CCR7, CCL18 and CD200R was performed using each primer (Supplementary Table 1). Beta 2-microglobulin of Universal ProbeLibrary (Roche Diagnostics) was used as a house-keeping control gene. Fold changes were depicted in mRNA levels after stimulation compared with unstimulated cells.

Using the iMG cells from a female NHD patient and an age-sex matched healthy control, we examined the gene expression of DAP12 and TREM2 by qRT-PCR. The iMG cells were washed and the total RNA was extracted respectively, and qRT-PCR was performed using each primer (Supplementary Table 1). Beta 2-microglobulin was used as a house-keeping control gene. Fold changes were depicted in mRNA levels after stimulation compared with unstimulated cells.

Phagocytosis. Phagocytosis was examined by fluorescent microscopy using Phagocytosis Assay Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. The iMG cells were cultured in 8-well chambers (Nalge Nunc International) at a density of 4×10^5 cells/ml. We added 50 μl of the latex beads-rabbit IgG-FITC solution to each well of the chamber, and incubated the cells in standard culture conditions for 24 hours. After discarding the supernatant by careful aspiration, we quenched surface-bound fluorescence, added 125 μl of trypan blue solution to each well of the chamber, and incubated for two minutes at room temperature. Each well was analyzed by using a fluorescence microscope (Olympus IX-71, Tokyo, Japan) and DP71 digital camera system (Olympus).

Cytokine measurement. Secretion of pro- and anti- inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8 and IL-10) during phagocytosis was measured from culture supernatants using Cytometric Beads Array System (CBA; BD Biosciences) according to the manufacturer's protocol. Latex beads-rabbit IgG-FITC solution (Cayman Chemical) was added to the iMG cells and incubated for 24 or 72 hours in standard culture conditions. After incubation, culture supernatants were centrifuged at 10000 × g for 10 minutes and kept frozen at −80°C until assayed. The culture supernatants were incubated with the cytokine capture beads and PE-conjugated detection antibodies for 3 hours at room temperature. Afterwards, the capture beads were washed and measurement data were acquired using a FACS Canto™ flow cytometer (BD Biosciences). The data analysis was performed using FCAP Array software (BD Biosciences).

Gene silencing of DAP12. Gene silencing assay was performed using siRNA (DAP12; Santa Cruz, USA) and RNAiMAX (Invitrogen) according to the manufacturer's protocol. The mix solution of siRNA and RNAiMAX was added to the iMG cells. After overnight incubation, the medium was changed to the culture medium and incubated for 48 hours. The siRNA-modified iMG cells were used for cytokine assay.

Statistical analysis. Analysis of comparisons between groups were conducted by two-tailed Student's t-test.

1. Ginhoux, F. *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* **330**, 841–845 (2010).
2. Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308**, 1314–1318 (2005).
3. Kettenmann, H., Hanisch, U. K., Noda, M. & Verkhratsky, A. Physiology of microglia. *Physiol Rev* **91**, 461–553 (2011).
4. Ransohoff, R. M. & Perry, V. H. Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol* **27**, 119–145 (2009).
5. Graeber, M. B. Changing face of microglia. *Science* **330**, 783–788 (2010).
6. Block, M. L., Zecca, L. & Hong, J. S. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci* **8**, 57–69 (2007).
7. Hanisch, U. K. & Kettenmann, H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* **10**, 1387–1394 (2007).
8. Kato, T. A. *et al.* Neurotransmitters, psychotropic drugs and microglia: clinical implications for psychiatry. *Curr Med Chem* **20**, 331–344 (2013).
9. Hakola, H. P. Neuropsychiatric and genetic aspects of a new hereditary disease characterized by progressive dementia and lipomembranous polycystic osteodysplasia. *Acta Psychiatr Scand Suppl* **232**, 1–173 (1972).
10. Nasu, T., Tsukahara, Y. & Terayama, K. A lipid metabolic disease-"membranous lipodystrophy"-an autopsy case demonstrating numerous peculiar membrane-structures composed of compound lipid in bone and bone marrow and various adipose tissues. *Acta Pathol Jpn* **23**, 539–558 (1973).
11. Kaneko, M., Sano, K., Nakayama, J. & Amano, N. Nasu-Hakola disease: The first case reported by Nasu and review. *Neuropathology* **30**, 463–470 (2010).
12. Paloneva, J. *et al.* CNS manifestations of Nasu-Hakola disease: a frontal dementia with bone cysts. *Neurology* **56**, 1552–1558 (2001).
13. Paloneva, J. *et al.* Loss-of-function mutations in TYROBP (DAP12) result in a presenile dementia with bone cysts. *Nat Genet* **25**, 357–361 (2000).
14. Paloneva, J. *et al.* Mutations in two genes encoding different subunits of a receptor signaling complex result in an identical disease phenotype. *Am J Hum Genet* **71**, 656–662 (2002).
15. Roumier, A. *et al.* Impaired synaptic function in the microglial KARAP/DAP12-deficient mouse. *J Neurosci* **24**, 11421–11428 (2004).
16. Satoh, J. *et al.* Immunohistochemical characterization of microglia in Nasu-Hakola disease brains. *Neuropathology* **31**, 363–375 (2011).
17. Mattis, V. B. & Svendsen, C. N. Induced pluripotent stem cells: a new revolution for clinical neurology? *Lancet Neurol* **10**, 383–394 (2011).
18. Dimos, J. T. *et al.* Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* **321**, 1218–1221 (2008).

19. Pfisterer, U. *et al.* Direct conversion of human fibroblasts to dopaminergic neurons. *Proc Natl Acad Sci U S A* **108**, 10343–10348 (2011).
20. Qiang, L. *et al.* Directed conversion of Alzheimer's disease patient skin fibroblasts into functional neurons. *Cell* **146**, 359–371 (2011).
21. Pang, Z. P. *et al.* Induction of human neuronal cells by defined transcription factors. *Nature* **476**, 220–223 (2011).
22. Wang, Y. *et al.* IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. *Nat Immunol* **13**, 753–760 (2012).
23. Aloisi, F., De Simone, R., Columba-Cabezas, S., Penna, G. & Adorini, L. Functional maturation of adult mouse resting microglia into an APC is promoted by granulocyte-macrophage colony-stimulating factor and interaction with Th1 cells. *J Immunol* **164**, 1705–1712 (2000).
24. Erblich, B., Zhu, L., Etgen, A. M., Dobrenis, K. & Pollard, J. W. Absence of colony stimulation factor-1 receptor results in loss of microglia, disrupted brain development and olfactory deficits. *PLoS One* **6**, e26317 (2011).
25. Nandi, S. *et al.* The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation. *Dev Biol* **367**, 100–113 (2012).
26. Sedgwick, J. D. *et al.* Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. *Proc Natl Acad Sci U S A* **88**, 7438–7442 (1991).
27. Melief, J. *et al.* Phenotyping primary human microglia: tight regulation of LPS responsiveness. *Glia* **60**, 1506–1517 (2012).
28. Mizutani, M. *et al.* The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. *J Immunol* **188**, 29–36 (2012).
29. Hinze, A. *et al.* Microglia differentiation using a culture system for the expansion of mice non-dadherent bone marrow stem cells. *J Inflamm* **9**, 12 (2012).
30. Noto, D. *et al.* Development of a culture system to induce microglia-like cells from haematopoitic cells. *Neuropathol Appl Neurobiol.* Doi10.1111/nan.12086. (2013).
31. Guilemini, G. *et al.* Granulocyte macrophage colony stimulating factor stimulates in vitro proliferation of astrocytes derived from simian mature brains. *Glia* **16**, 71–80 (1996).
32. Gomez-Nicola, D. *et al.* Regulation of microglial proliferation during chronic neurodegeneration. *J Neurosci* **33**, 2481–2493 (2013).
33. Schwarz, J. M. & Bilbo, S. D. Sex, glia, and development: interactions in health and disease. *Horm Behgav* **62**, 243–253 (2012).
34. Paradowska-Gorycka, A. & Jurkowska, M. Structure, expression pattern and biological activity of molecular complex TREM-2/DAP12. *Hum Immunol* **74**, 730–737 (2013).
35. Hamerman, J. A., Tchao, N. K., Lowell, C. A. & Lanier, L. L. Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. *Nat Immunol* **6**, 579–586 (2005).

Acknowledgments

The authors would like to thank Ms. Mayumi Tanaka and Ms. Mayumi Inenaga for their technical assistances. We appreciate the technical support from Department of Dermatology (Prof. Masutaka Furue) and the Research Support Center, Graduate School of Medical Sciences, Kyushu University. This work was supported by Grant-in-Aid for Scientific Research on (1) the Japan Society for the Promotion of Science (to TAK, MO and SK), (2) Innovative Areas "Glia Assembly" of The Ministry of Education, Culture, Sports, Science, and Technology, Japan (No. 25117011 to SK), (3) the Health and Labour Sciences Research Grant (No. (H24-Seishin-Jitsuyouka (Seishin)-Ippan-001 to SK), (4) Young Principal Investigators' Research Grant of Innovation Center for Medical Redox Navigation, Kyushu University (to TAK), (5) Takeda Science Foundation – Medical Research (to TAK), and (6) SENSHIN Medical Research Foundation (to TAK). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

All authors contributed substantially to the scientific process leading up to the writing of the present paper. T.A.K., the principal investigator of the present research, and M.O., the first author, created the conception and design of the project and wrote the protocol. The performance of experiments and the data analysis/interpretation were performed by M.O., T.A.K., D.S., N.S., K.H. and N.S. M.O. wrote the first draft of the manuscript. Clinical recruitments were conducted by R.H., K.S., T.Y., K.H. and N.S. Critical revisions of the manuscript were made by T.A.K., D.M., H.U. and S.K. All authors have approved to submit the final manuscript.

Additional information

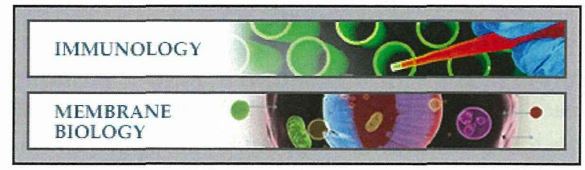
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Ohgidani, M. *et al.* Direct induction of ramified microglia-like cells from human monocytes: Dynamic microglial dysfunction in Nasu-Hakola disease. *Sci. Rep.* 4, 4957; DOI:10.1038/srep04957 (2014).

Immunology:

Brain-derived Neurotrophic Factor (BDNF) Induces Sustained Intracellular Ca^{2+} Elevation through the Up-regulation of Surface Transient Receptor Potential 3 (TRPC3) Channels in Rodent Microglia



Yoshito Mizoguchi, Takahiro A. Kato, Yoshihiro Seki, Masahiro Ohgidani, Noriaki Sagata, Hideki Horikawa, Yusuke Yamauchi, Mina Sato-Kasai, Kohei Hayakawa, Ryuji Inoue, Shigenobu Kanba and Akira Monji

J. Biol. Chem. 2014, 289:18549-18555.
doi: 10.1074/jbc.M114.555334 originally published online May 8, 2014

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 26, pp. 18549–18555, June 27, 2014

Brain-derived Neurotrophic Factor (BDNF) Induces Sustained Intracellular Ca^{2+} Elevation through the Up-regulation of Surface Transient Receptor Potential 3 (TRPC3) Channels in Rodent Microglia*

Received for publication, February 1, 2014 Published, JBC Papers in Press, May 8, 2014, DOI 10.1074/jbc.M114.555334

Yoshito Mizoguchi[‡1], **Takahiro A. Kato**[§¶], **Yoshihiro Seki**[§], **Masahiro Ohgidani**[§¶], **Noriaki Sagata**[§¶], **Hideki Horikawa**[§], **Yusuke Yamauchi**[§], **Mina Sato-Kasai**[§], **Kohei Hayakawa**[§], **Ryuji Inoue**[‖], **Shigenobu Kanba**[§], **and Akira Monji**[‡]

From the [‡]*Department of Psychiatry, Faculty of Medicine, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan, the* [§]*Department of Neuropsychiatry, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan, the* [¶]*Innovation Center for Medical Redox Navigation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan, and the* [‖]*Department of Physiology, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jyonan-ku, Fukuoka 812-0180, Japan*

Background: BDNF and Ca^{2+} mobilization is important for microglial function.
Results: We showed BDNF elevates intracellular Ca^{2+} through TRPC3 channels.
Conclusion: TRPC3 is important for BDNF suppression of microglial activation.
Significance: TRPC3 might be important for the treatment of psychiatric disorders.

Microglia are immune cells that release factors, including proinflammatory cytokines, nitric oxide (NO), and neurotrophins, following activation after disturbance in the brain. Elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is important for microglial functions such as the release of cytokines and NO from activated microglia. There is increasing evidence suggesting that pathophysiology of neuropsychiatric disorders is related to the inflammatory responses mediated by microglia. Brain-derived neurotrophic factor (BDNF) is a neurotrophin well known for its roles in the activation of microglia as well as in pathophysiology and/or treatment of neuropsychiatric disorders. In this study, we sought to examine the underlying mechanism of BDNF-induced sustained increase in $[Ca^{2+}]_i$ in rodent microglial cells. We observed that canonical transient receptor potential 3 (TRPC3) channels contribute to the maintenance of BDNF-induced sustained intracellular Ca^{2+} elevation. Immunocytochemical technique and flow cytometry also revealed that BDNF rapidly up-regulated the surface expression of TRPC3 channels in rodent microglial cells. In addition, pretreatment with BDNF suppressed the production of NO induced by tumor necrosis factor α (TNFα), which was prevented by co-adiministration of a selective TRPC3 inhibitor. These suggest that BDNF induces sustained intracellular Ca^{2+} elevation through the up-regulation of surface TRPC3 channels and TRPC3 channels could be important for the BDNF-induced suppression of the NO production in activated microglia. We show that TRPC3 channels could also play important roles in microglial functions, which might be important for the regulation of inflammatory responses and may also be involved in the pathophysiology and/or the treatment of neuropsychiatric disorders.

Microglia are immune cells that release proinflammatory cytokines, nitric oxide (NO), and neurotrophins, when they are activated in response to brain injury or immunological stimuli (1). There is increasing evidence suggesting that pathophysiology of neuropsychiatric disorders is related to inflammatory responses mediated by microglial cells (2, 3).

In the rodent brain, microglial cells secrete brain-derived neurotrophic factor (BDNF), and BDNF promotes the proliferation and survival of microglia themselves (4). In addition, pretreatment with BDNF suppressed the release of NO from murine microglial cells activated by IFN-γ (5). To date, BDNF is also well known for its involvement in the pathophysiology of neuropsychiatric disorders (4, 5).

Elevation of intracellular Ca^{2+} is important in activation of microglial cell functions, including proliferation, release of NO, and migration (1). We have reported previously that BDNF induces a sustained increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through the activation of the phospholipase C (PLC)[2] pathway in rodent microglial cells (5). We also tested the effect of 2-aminoethoxydiphenyl borate or SKF-96365, both of which can inhibit canonical transient receptor potential (TRPC) channels (6, 7) and showed that sustained activation of TRPC channels occurred after a brief treatment with BDNF and contributed to the maintenance of BDNF-induced sustained intracellular Ca^{2+} elevation (5).

In this study, we examined whether TRPC3 channels contribute to the maintenance of BDNF-induced sustained intra-

* This work was supported by research grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to Y. M., T. A. K., S. K., and A. M.).

[1] To whom correspondence should be addressed: Dept. of Neuropsychiatry, Faculty of Medicine, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan. Tel.: 81-952-34-2304; Fax: 81-952-34-2048; E-mail: ymizo@pk2.so-net.ne.jp.

[2] The abbreviations used are: PLC, phospholipase C; TRPC3, canonical transient receptor potential 3; Pyr3, pyrazole compound 3; HAPI, highly aggressive proliferating immortalized; DAF, 4,5-diaminofluorescein.

cellular Ca^{2+} elevation using the pyrazole compound 3 (Pyr3), a selective inhibitor of TRPC3 channels, which does not affect the activity of other TRPC channel members (8, 9), in rodent microglial cells. Although mRNAs of many TRPC channels, including TRPC3, are shown to be expressed in cultured rat microglia (10), this is the first report showing that TRPC3 channels could also play important roles in microglial functions.

EXPERIMENTAL PROCEDURES

Materials—The drugs used in the present study include Fura2-AM, 4,5-diaminofluorescein diacetate, U73122, and human recombinant TNFα (from Sigma) and polyclonal rabbit anti-TRPC3 channel antibody (ACC-016; Alomone Labs, Jerusalem, Israel). Recombinant IFN-γ and mouse GM-CSF were purchased from R&D Systems. Human recombinant BDNF (Sigma) was diluted with the standard external solution to obtain the final concentration (20 ng/ml; 0.73 nM), which is sufficient to promote the proliferation of microglial cells (4, 5). The final concentration of dimethyl sulfoxide was always <0.1%.

Microglial Cells—Primary microglial cells were prepared from the whole brain of 3-day postnatal Sprague-Dawley rats as described previously (5, 11, 12). Primary mixed cells were prepared from the whole brain of 3-day-postnatal Sprague-Dawley rats using a Cell Strainer (BD Biosciences). Primary rat microglial cells were selected after attachment to Aclar film (Nisshin EM) for 2 h in DMEM supplemented with 10% FBS (10% FBS/DMEM). Aclar films were gently washed with PBS and then transferred to fresh 10% FBS/DMEM, and the fresh microglia expanded for 1–2 days. The purity of isolated microglia was assessed by immunocytochemical staining for the microglial marker, Iba-1, and >99% of cells stained positively (13, 14). The 6-3 microglial cells were established from neonatal C57BL/6J (H-2b) mice as described previously (5, 11–14).

The 6-3 cells were cultured in Eagle's minimal essential medium supplemented with 0.3% $NaHCO_3$, 2 mM glutamine, 0.2% glucose, 10 g/ml insulin, and 10% FCS. Cells were maintained at 37 °C in a 10% CO_2, 90% air atmosphere. GM-CSF was supplemented into the culture medium, at a final concentration of 1 ng/ml, to maintain proliferation of the 6-3 cells. Culture medium was renewed twice per week.

The rat microglial cell line, highly aggressive proliferating immortalized (HAPI) cells (15), was kindly donated by Drs. N. P. Morales and F. Hyodo of Kyushu University. The cells were cultured in DMEM (low glucose; Invitrogen), 5% FBS (Hyclone), 4 mM glutamine (Invitrogen), 100 000 units/liter penicillin G, 100 mg/liter streptomycin (Mediatech), and maintained in 5% CO_2 at 37 °C.

siRNA Transfection—To down-regulate TRPC3 channels, siRNA transfection was performed. The 6-3 microglial cells were cultured in growth medium without antibiotics in a 35-mm glass-based dish until 60–80% confluent. TRPC3-targeting siRNA or scrambled control siRNA (sc-42667 and sc-37007, 80 pmol/dish, respectively; Santa Cruz Biotechnology, Santa Cruz, CA) were transfected into 6-3 microglial cells using siRNA transfection reagent (sc-29528, 6 μl/dish; Santa Cruz Biotechnology) in siRNA transfection medium (sc-36868, 1 ml/dish; Santa Cruz Biotechnology) according to the manufacturer's instructions. Six hours after transfection, normal serum and antibiotics were added at final concentrations of 10 and 1%, respectively. The next day, the medium containing transfection mixtures was replaced with fresh growth medium. At 48 h, the transfected cells were used for intracellular Ca^{2+} imaging.

Intracellular Ca^{2+} Imaging—Intracellular Ca^{2+} imaging using Fura-2-AM was performed as reported previously (5, 16, 17). In brief, the experiments were performed in the external standard solution (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4, with Tris-OH) at room temperature (27 °C). For Fura-2 excitation, the cells were illuminated with two alternating wavelengths, 340 and 380 nm using a computerized system for a rapid dual wavelength Xenon arc. The emitted light was recorded at 510 nm using a cooled CCD camera (Hamamatsu Photonics). The $[Ca^{2+}]_i$ was calculated from the ratio (R) of fluorescence recorded at 340 and 380 nm excitation wavelengths for each pixel within a microglial cell boundary. All data presented were obtained from at least five dishes and three different cell preparations.

Immunocytochemistry—After microglial cells were fixed, indirect immunofluorescence was performed using the following antibodies: polyclonal rabbit anti-TRPC3 channel antibody (ACC-016; Alomone Labs, Jerusalem, Israel), which recognizes intracellular C terminus of mouse TRPC3 channel and mouse anti-CD45 monoclonal antibody. These specimens were incubated in primary antibodies, and FITC- or Texas red-conjugated secondary antibodies were used for detection. Fluorescent images were captured with a fluorescence microscope (Axio Scope A1, Carl Zeiss, Oberkochen, Germany).

Flow Cytometry—Flow cytometry was performed using a FACS Canto II (BD Biosciences) with FACS Diva software (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star, San Carios, CA). The HAPI microglial cells were harvested by non-enzymatic cell dissociation solution (Sigma) and cell lifter (Corning). The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking, the cells were stained with anti-TRPC3 antibody (ACC-016). After washing, the cells were stained with Alexa Fluor 488-conjugated secondary antibody (Invitrogen). The fluorescence intensity of the cells was measured.

Intracellular NO Imaging—The microglial cells were loaded with 10 μM 4,5-diaminofluorescein diacetate (Sigma), a cell membrane-permeable dye that binds intracellular NO (18), for 20 min before the measurement. For DAF-2 excitation, the cells were illuminated with a wavelength, 490 nm, using a computerized system. The signal obtained at 490 nm was previously shown to be, among the excitation wavelengths, quantitatively largest and most representative of change in intracellular NO (19). The emitted light was collected at 510 nm using a cooled CCD camera. The intracellular DAF-2 fluorescence intensity (F) was recorded for each pixel within a cell boundary. The ratio (F/F_0) of fluorescence intensity was estimated from the intensity of fluorescence recorded prior to stimulation (F_0).

All data are expressed as the mean ± S.E., and statistical comparisons were made using an unpaired t test. Significance was established at a level of $p < 0.05$.

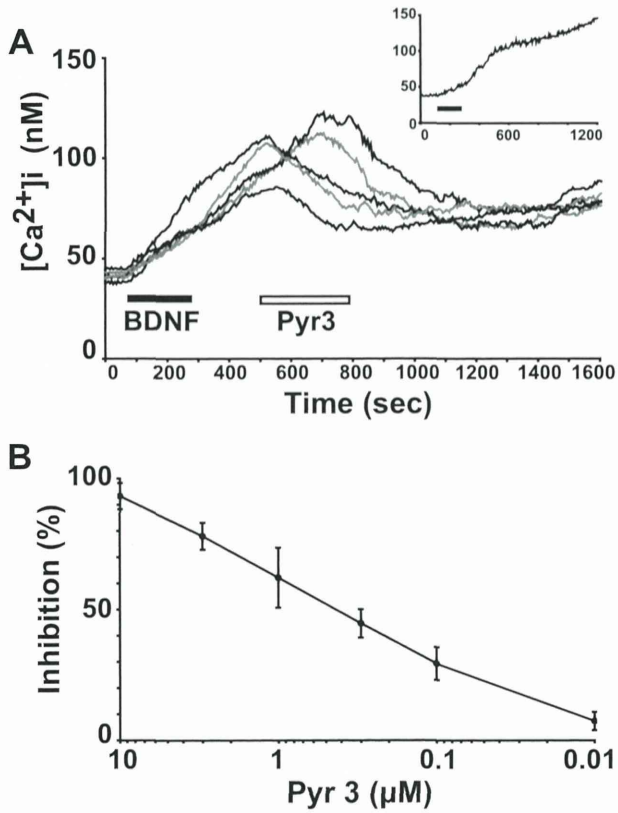


FIGURE 1. **TRPC3 channels contribute to the maintenance of BDNF-induced sustained intracellular Ca^{2+} elevation in rodent microglial cells.** *A*, five representative traces showing the effect of 0.3 μM Pyr3, a selective inhibitor of TRPC3 channels, after the onset of 20 ng/ml BDNF-induced sustained elevation of $[Ca^{2+}]_i$ in primary rat microglial cells. *Inset*, the inset shows a brief (3 min) treatment of BDNF-induced sustained increase in $[Ca^{2+}]_i$ in primary rat microglial cells. The average trace of five $[Ca^{2+}]_i$ traces in response to BDNF is shown. *B*, the dose-response effect of different concentrations of Pyr3 on inhibition of the amplitude of $[Ca^{2+}]_i$ increase obtained 15 min after BDNF treatment in primary rat microglial cells. Values are the mean ± S.E.

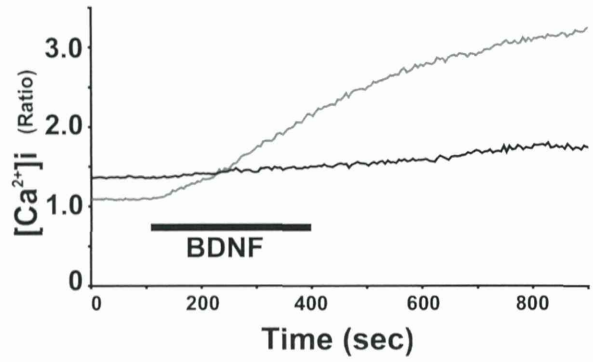


FIGURE 2. **Down-regulation of TRPC3 channels abolished the BDNF-induced elevation of intracellular Ca^{2+} in rodent microglial cells.** The average traces showing the effect of BDNF (20 ng/ml) on intracellular Ca^{2+} mobilization in 6-3 microglial cells transfected with TRPC3-targeting siRNA (*black line*) and scrambled control siRNA (*gray line*). The average trace was determined from 10 representative traces of intracellular Ca^{2+} in each condition.

RESULTS

We have previously reported that BDNF induces sustained increase in intracellular Ca^{2+} in rodent microglial cells (Fig. 1*A*, *inset*) (5). The increase in intracellular Ca^{2+} was sustained for >40 min even after the washout of BDNF until the end of recording. We applied the Pyr3, a selective inhibitor of TRPC3 channels (8, 9), after the onset of BDNF-induced sustained intracellular Ca^{2+} elevation to investigate the involvement of TRPC3 channels in the maintenance of long lasting $[Ca^{2+}]_i$ elevation. After the onset of BDNF-induced intracellular Ca^{2+} elevation, Pyr3 (0.3 μM) was applied and found to suppress the $[Ca^{2+}]_i$ in the 6-3 (n = 35 cells; data not shown) and primary (n = 78 cells; Fig. 1*A*) microglial cells. As shown in Fig. 1*B*, application of Pyr3 suppressed BDNF-induced intracellular Ca^{2+} elevation in a dose-dependent manner with the IC_{50} value of 0.5 μM. We observed that 10 μM Pyr3 suppressed the $[Ca^{2+}]_i$ to near basal levels in the 6-3 (n = 22) and primary (n = 21) microglial cells.

To confirm the involvement of TRPC3 channels in the BDNF-induced increase in $[Ca^{2+}]_i$, we down-regulated TRPC3 protein expression using siRNA. As expected, down-regulation of TRPC3 with siRNA suppressed the elevation of $[Ca^{2+}]_i$ induced by BDNF (Fig. 2). These indicate that sustained activation of TRPC3 channels could occur after a brief application of BDNF and contribute to the maintenance of BDNF-induced sustained intracellular Ca^{2+} elevation in rodent microglial cells.

Next, we performed immunocytochemistry to examine the association between TRPC3 surface expression and BDNF in rodent microglia. RT-PCR analysis has shown previously that TRPC3 mRNA is expressed in cultured microglial cells derived from rats (10). We also confirmed the same results in primary microglial cells and 6-3 murine microglial cells (data not shown). Although only weak TRPC3 immunoreactivity was observed in somata of control HAPI microglial cells, a dramatic increase in TRPC3 expression was observed in BDNF-treated HAPI microglial cells (Fig. 3, *A* and *B*). Double immunostaining for TRPC3 and CD45 (cytoplasmic staining of immune cells) demonstrated that TRPC3 was strongly stained on the cell surface of HAPI microglial cells after the BDNF application, suggesting that BDNF rapidly up-regulated the surface expression of TRPC3 channels in rodent microglial cells (Fig. 3, *C* and *D*).

To quantify the above-mentioned results, we next examine the effect of BDNF on surface expression of TRPC3 channels in HAPI microglial cells using flow cytometry. We observed that BDNF rapidly increased the relative expression of surface TRPC3 channels in HAPI microglial cells (n = 3; Fig. 4). Altogether, these indicate that BDNF induces sustained intracellular Ca^{2+} elevation possibly through the up-regulation of surface TRPC3 channels in rodent microglial cells.

We have previously shown that the activation of PLC is involved in the induction of BDNF-induced intracellular Ca^{2+} elevation in rodent microglial cells (5). In the next examination, we observed that pretreatment of U73122 (5 μM), a membrane-permeable specific PLC inhibitor, significantly reduced the amplitude of BDNF-induced increase in relative expression of surface TRPC3 channels in HAPI microglial cells (n = 3; Fig. 4). Thus, the activation of PLC could also be important for the up-regulation of surface TRPC3 channels induced by BDNF in rodent microglial cells.

We have previously reported that pretreatment with BDNF suppressed the release of NO from murine microglial cells activated by IFN-γ (5). In addition, pretreatment of BDNF suppressed the IFN-γ-induced elevation of $[Ca^{2+}]_i$, along with a

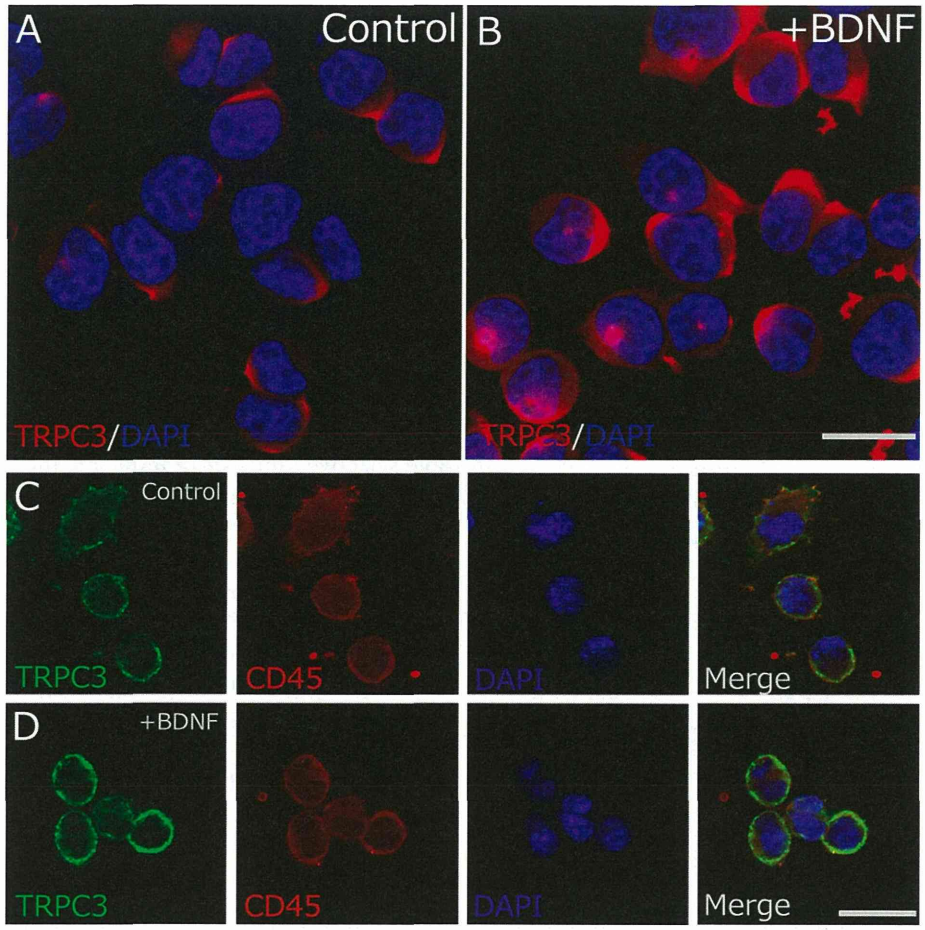


FIGURE 3. **BDNF up-regulates the surface expression of TRPC3 channels in rodent microglial cells.** TRPC3 (*red*) is markedly up-regulated in the BDNF-treated (20 ng/ml, 10 min) HAPI cells (*B*) compared with control cells (*A*). *C* and *D*, two representative confocal images of HAPI microglial cells showing substantial staining of TRPC3 (*green*) and CD45 (*red*). The surface expression of TRPC3 is up-regulated in BDNF-treated HAPI cells (*D*) compared with control cells (*C*). The nuclei are stained with 4′,6-diamidino-2-phenylindole (*blue*). The *scale bars* indicate 20 μm.

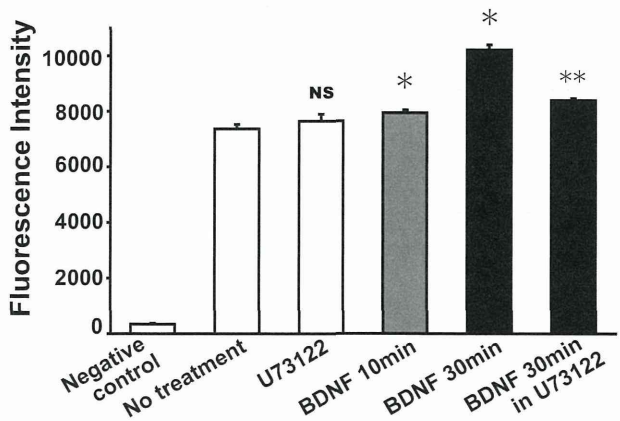


FIGURE 4. **Quantification of the effect of BDNF on the surface expression of TRPC3 channels in rodent microglial cells.** Flow cytometry showing that treatment of BDNF (20 ng/ml) rapidly increased the fluorescence intensity of surface expression of TRPC3 channels in HAPI microglial cells. In contrast, pretreatment of U73122 (5 μM), a membrane-permeable specific PLC inhibitor, significantly reduced the amplitude of BDNF-induced increase in the expression of surface TRPC3 channels in HAPI microglial cells. *, $p < 0.05$ *versus* no treatment; **, $p < 0.01$ *versus* BDNF (30 min). Negative control was obtained from secondary antibody alone. *NS,* nonsignificant.

rise in basal $[Ca^{2+}]_i$ in rodent microglial cells (5). Thus, BDNF-induced elevation of basal levels of $[Ca^{2+}]_i$ could regulate the microglial intracellular signal transduction to suppress the release of NO induced by IFN-γ (4, 5). We next tested whether TRPC3 channels could be important for the BDNF-induced suppression of NO production in rodent microglial cells.

In the present study, 50 units/ml IFN-γ induced sustained intracellular Ca^{2+} elevation in both 6-3 and primary microglial cells as reported previously (data not shown) (5). After the onset of IFN-γ-induced intracellular Ca^{2+} elevation, 3 μM Pyr3 was applied and found to suppress the $[Ca^{2+}]_i$ to near basal levels in the 6-3 ($n = 24$; data not shown) and primary ($n = 47$; Fig. 5*A*) microglial cells. Thus, TRPC3 channels could also contribute to the maintenance of IFN-γ-induced sustained intracellular Ca^{2+} elevation in rodent microglial cells used in this study.

TNFα, one of the proinflammatory cytokines, was shown to induce a gradual increase in intracellular Ca^{2+} in cultured astrocytes at a concentration of 2 μg/ml (20). In the present study, 2 μg/ml TNFα rapidly increased $[Ca^{2+}]_i$ in both 6-3 ($n = 23$; data not shown) and primary microglial cells ($n = 41$; data not shown). Once the intracellular Ca^{2+} level rose, it gradually increased without attenuation even after the wash-out of TNFα until the end of recording. Interestingly, 3 μM Pyr3 applied after the onset of TNFα-induced intracellular Ca^{2+} elevation did not affect $[Ca^{2+}]_i$ in 6-3 ($n = 21$) and primary ($n = 58$; Fig. 5*B*) microglial cells. These suggest that TRPC3 channels could not be important for the mainte-

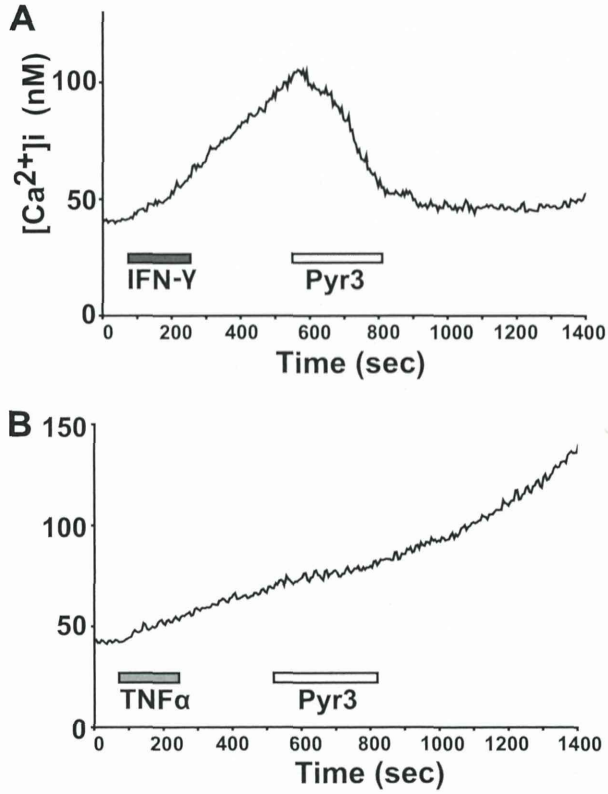


FIGURE 5. **TRPC3 channels are not involved in TNFα-induced sustained intracellular Ca^{2+} elevation in rodent microglial cells.** *A* and *B*, an average trace showing the effect of the 3 μM Pyr3 after the onset of 50 units/ml IFN-γ-induced or 2 μg/ml TNFα-induced sustained elevation of $[Ca^{2+}]_i$ in primary rat microglial cells. Each panel demonstrates the average trace determined from 10 representative traces of $[Ca^{2+}]_i$ in each condition.

nance of TNFα-induced sustained intracellular Ca^{2+} elevation in rodent microglial cells we used.

We next tested the effect of TNFα on intracellular NO mobilization, using DAF-2 imaging to detect endogenously produced NO in rodent microglia. An application of 2 μg/ml TNFα induced a gradual increase in DAF-2 fluorescence in both 6-3 ($n = 101$; Fig. 6*A*) and primary ($n = 45$; data not shown) microglial cells tested. The reaction between DAF-2 and NO is shown to be irreversible and the accumulated level of DAF-2 fluorescence reflects the total amount of intracellular NO production (18, 21). We observed that the increase in intracellular DAF-2 fluorescence was sustained for > 40 min even after the washout of TNFα until the end of recording. Additionally, in the presence of 50 μM L-N6-(1-iminoethyl)lysine, a membrane-permeable selective inhibitor of inducible nitric oxide synthase (22), TNFα failed to elevate the DAF-2 fluorescence in both 6-3 ($n = 43$) and primary ($n = 11$) microglial cells (data not shown).

We measured the effect of 24-h pretreatment with BDNF (20 ng/ml) on the production of intracellular NO induced by TNFα in rodent microglia. In 6-3 microglial cells that were pretreated with BDNF for 24 h, TNFα (2 μg/ml) also induced a gradual increase in the DAF-2 fluorescence (Fig. 6*B*). However, pretreatment of BDNF significantly reduced the amplitude of TNFα-induced increase in the DAF-2 fluorescence at 15 min after a treatment of TNFα in 6-3 microglial cells (0.171 ± 0.019, $n = 101$ in control; 0.019 ± 0.007, $n = 27$ in 5 ng/ml BDNF; 0.018 ± 0.006, $n = 68$ in 20 ng/ml BDNF; $p < 0.001$; Fig. 6*D*). In contrast, 24 h pretreatment of both BDNF (20 ng/ml) and Pyr3 (0.2 μM) did not reduce the amplitude of TNFα-induced increase in the DAF-2 fluorescence in 6-3 microglial cells

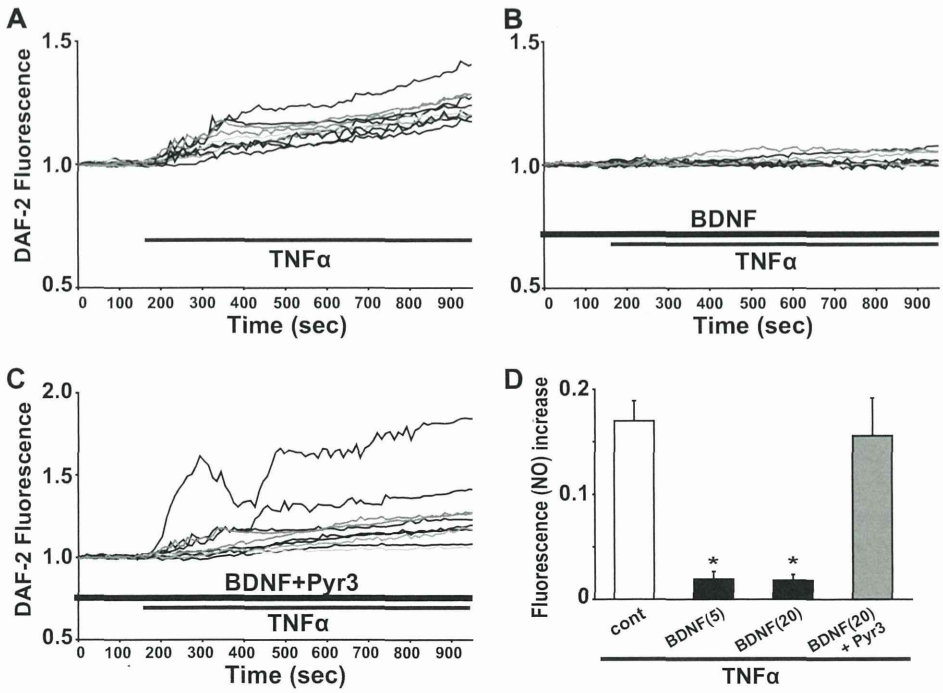


FIGURE 6. **Pretreatment with BDNF suppressed the production of NO induced by TNFα in 6-3 microglial cells.** *A*, 10 representative traces showing the treatment of 2 μg/ml TNFα induced the increase in the DAF-2 fluorescence in murine 6-3 microglial cells. *B*, 10 representative traces showing 24-h pretreatment with 20 ng/ml BDNF suppressed the TNFα-induced increase in the DAF-2 fluorescence in murine 6-3 microglial cells. *C*, 10 representative traces showing 24-h pretreatment with both 20 ng/ml BDNF and 0.2 μM Pyr3 did not suppress the TNFα-induced increase in the DAF-2 fluorescence in murine 6-3 microglial cells. *D*, bar graphs showing that pretreatment with BDNF suppressed the production of NO induced by TNFα treatment and TRPC3 channels could be important for the BDNF-induced suppression of the NO production in murine 6-3 microglial cells. BDNF (5) and BDNF (20) mean 5 ng/ml BDNF and 20 ng/ml BDNF for each.

(0.171 ± 0.019, n = 101 in control; 0.156 ± 0.036, n = 69 in BDNF + Pyr3; p = 0.37; Fig. 6, *C* and *D*). These suggest that pretreatment with BDNF suppressed the production of NO induced by TNFα. In addition, TRPC3 channels could be important for the BDNF-induced suppression of NO production in rodent microglial cells.

DISCUSSION

We found that TRPC3 channels mainly contributed to the maintenance of BDNF-induced sustained intracellular Ca^{2+} elevation in rodent microglial cells. In addition, we suggest that TRPC3 channels could be important for BDNF-induced suppression of NO production in rodent microglial cells activated by TNFα.

BDNF-induced elevation of basal levels of $[Ca^{2+}]_i$ could regulate the microglial intracellular signal transduction to suppress the release of NO induced by IFN-γ (4, 5, 23). We herein showed that pretreatment with BDNF also suppressed the production of NO in murine microglial cells activated by TNFα, which was prevented by co-administration of Pyr3. We also found that pretreatment with both BDNF and Pyr3 did not elevate the basal $[Ca^{2+}]_i$ in rodent microglial cells (data not shown). These suggest that BDNF-induced elevation of basal levels of $[Ca^{2+}]_i$ mediated by TRPC3 channels could be important for the BDNF-induced suppression of NO production in rodent microglial cells.

We observed an application of Pyr3 did not suppress the elevation of $[Ca^{2+}]_i$ induced by TNFα in rodent microglial cells. TRPM2 channels, a member of the melastatin subfamily of TRP channels, are shown to mediate the TNFα-induced intracellular $[Ca^{2+}]_i$ oscillation (24), suggesting that TRPM2 channels might be involved in the TNFα-induced sustained $[Ca^{2+}]_i$ increase in rodent microglial cells.

We have recently reported that pretreatment with antidepressants (13) or antipsychotics (14, 25) significantly inhibits the release of NO from activated microglia. In this study, we observed that pretreatment with BDNF significantly inhibited the production of NO in microglia activated by TNFα. TNFα plays a key role in the induction of sickness behaviors (26) and also in the development of depressive symptoms (27). Thus, this would suggest that BDNF might have an anti-inflammatory effect through the inhibition of microglial activation and could be useful for the treatment of neuropsychiatric disorders. We need to further examine the mechanism underlying the up-regulation of surface TRPC3 channels induced by BDNF in rodent microglial cells.

Acknowledgments—We thank Drs. Shigeki Kiyonaka and Yasuo Mori of Kyoto University for providing us with the pyrazole compound 3 and also thank Dr. Makoto Sawada of Nagoya University for providing us with the microglial cell line, 6-3.

REFERENCES

1. Kettenmann, H., Hanisch, U. K., Noda, M., and Verkhratsky, A. (2011) Physiology of microglia. *Physiol. Rev.* **91,** 461–553
2. Monji, A., Kato, T. A., Mizoguchi, Y., Horikawa, H., Seki, Y., Kasai, M., Yamauchi, Y., Yamada, S., and Kanba, S. (2013) Neuroinflammation in schizophrenia especially focused on the role of microglia. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **42,** 115–121
3. Kato, T. A., Yamauchi, Y., Horikawa, H., Monji, A., Mizoguchi, Y., Seki, Y., Hayakawa, K., Utsumi, H., and Kanba, S. (2013) Neurotransmitters, psychotropic drugs and microglia: clinical implications for psychiatry. *Curr. Med. Chem.* **20,** 331–344
4. Mizoguchi, Y., Monji, A., Kato, T. A., Horikawa, H., Seki, Y., Kasai, M., Kanba, S., and Yamada, S. (2011) Possible role of BDNF-induced microglial intracellular Ca^{2+} elevation in the pathophysiology of neuropsychiatric disorders. *Mini Rev. Med. Chem.* **11,** 575–581
5. Mizoguchi, Y., Monji, A., Kato, T., Seki, Y., Gotoh, L., Horikawa, H., Suzuki, S. O., Iwaki, T., Yonaha, M., Hashioka, S., and Kanba, S. (2009) Brain-derived neurotrophic factor induces sustained elevation of intracellular Ca^{2+} in rodent microglia. *J. Immunol.* **183,** 7778–7786
6. Amaral, M. D., and Pozzo-Miller, L. (2007) TRPC3 channels are necessary for brain-derived neurotrophic factor to activate a nonselective cationic current and to induce dendritic spine formation. *J. Neurosci.* **27,** 5179–5189
7. Bootman, M. D., Collins, T. J., Mackenzie, L., Roderick, H. L., Berridge, M. J., and Peppiatt, C. M. (2002) 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of $InsP_3$-induced Ca^{2+} release. *FASEB J.* **16,** 1145–1150
8. Kiyonaka, S., Kato, K., Nishida, M., Mio, K., Numaga, T., Sawaguchi, Y., Yoshida, T., Wakamori, M., Mori, E., Numata, T., Ishii, M., Takemoto, H., Ojida, A., Watanabe, K., Uemura, A., Kurose, H., Morii, T., Kobayashi, T., Sato, Y., Sato, C., Hamachi, I., and Mori, Y. (2009) Selective and direct inhibition of TRPC3 channels underlies biological activities of a pyrazole compound. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 5400–5405
9. Kim, M. S., Lee, K. P., Yang, D., Shin, D. M., Abramowitz, J., Kiyonaka, S., Birnbaumer, L., Mori, Y., and Muallem, S. (2011) Genetic and pharmacologic inhibition of the Ca^{2+} influx channel TRPC3 protects secretory epithelia from Ca^{2+}-dependent toxicity. *Gastroenterology* **140,** 2107–2115
10. Ohana, L., Newell, E. W., Stanley, E. F., and Schlichter, L. C. (2009) The Ca^{2+} release-activated Ca^{2+} current (I(CRAC)) mediates store-operated Ca^{2+} entry in rat microglia. *Channels* **3,** 129–139
11. Kato, T., Mizoguchi, Y., Monji, A., Horikawa, H., Suzuki, S. O., Seki, Y., Iwaki, T., Hashioka, S., and Kanba, S. (2008) Inhibitory effects of aripiprazole on interferon-γ-induced microglial activation via intracellular Ca^{2+} regulation *in vitro*. *J. Neurochem.* **106,** 815–825
12. Seki, Y., Suzuki, S. O., Masui, K., Harada, S., Nakamura, S., Kanba, S., and Iwaki, T. (2011) A simple and high-yield method for preparation of rat microglial cultures utilizing Aclar plastic film. *Neuropathology* **31,** 215–222
13. Horikawa, H., Kato, T. A., Mizoguchi, Y., Monji, A., Seki, Y., Ohkuri, T., Gotoh, L., Yonaha, M., Ueda, T., Hashioka, S., and Kanba, S. (2010) Inhibitory effects of SSRIs on IFN-γ induced microglial activation through the regulation of intracellular calcium. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **34,** 1306–1316
14. Kato, T. A., Monji, A., Yasukawa, K., Mizoguchi, Y., Horikawa, H., Seki, Y., Hashioka, S., Han, Y. H., Kasai, M., Sonoda, N., Hirata, E., Maeda, Y., Inoguchi, T., Utsumi, H., and Kanba, S. (2011) Aripiprazole inhibits superoxide generation from phorbol-myristate-acetate (PMA)-stimulated microglia *in vitro*: implication for antioxidative psychotropic actions via microglia. *Schizophr. Res.* **129,** 172–182
15. Cheepsunthorn, P., Radov, L., Menzies, S., Reid, J., and Connor, J. R. (2001) Characterization of a novel brain-derived microglial cell line isolated from neonatal rat brain. *Glia* **35,** 53–62
16. Mizoguchi, Y., Ishibashi, H., and Nabekura, J. (2003) The action of BDNF on $GABA_A$ currents changes from potentiating to suppressing during maturation of rat hippocampal CA1 pyramidal neurons. *J. Physiol.* **548,** 703–709
17. Mizoguchi, Y., Kanematsu, T., Hirata, M., and Nabekura, J. (2003) A rapid increase in the total number of cell surface functional GABAA receptors induced by brain-derived neurotrophic factor in rat visual cortex. *J. Biol. Chem.* **278,** 44097–44102
18. Kojima, H., Nakatsubo, N., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H., Hirata, Y., and Nagano, T. (1998) Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal. Chem.* **70,** 2446–2453