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

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**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-administration 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-

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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.
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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 Fura-2-AM, 4,5-diaminofluorescein diacetate, U73122, and human recombinant TNF\(\alpha\) (from Sigma) and polyclonal rabbit anti-TRPC3 channel antibody (ACC-016; Alomone Labs, Jerusalem, Israel). Recombinant IFN-\(\gamma\) 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 \(\mu\)M), 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 selected after attachment to Aclar film (Nissin 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 \(\mu\)M 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 established at a level of 0.2% glucose, 10 g/ml insulin, and 10% FCS. Media 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, supplemented in 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.

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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.

Intracellular NO Imaging—The microglial cells were loaded with 10 \(\mu\)M 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 (I) was recorded for each pixel within a cell boundary. The ratio (I/I\(_{0}\)) of fluorescence intensity was estimated from the intensity of fluorescence recorded prior to stimulation (I\(_{0}\)).

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 \(p < 0.05\).
**RESULTS**

We have previously reported that BDNF induces sustained increase in intracellular Ca\(^{2+}\) in rodent microglial cells (Fig. 1A, 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\(^{2+}\)]\(\text{e}\) elevation. After the onset of BDNF-induced intracellular Ca\(^{2+}\) elevation, Pyr3 (0.3 \(\mu\)M) was applied and found to suppress the [Ca\(^{2+}\)]\(\text{e}\) in the 6-3 (\(n = 35\) cells; data not shown) and primary (\(n = 78\) cells; Fig. 1A) microglial cells. As shown in Fig. 1B, application of Pyr3 suppressed BDNF-induced intracellular Ca\(^{2+}\) elevation in a dose-dependent manner with the IC\(_{50}\) value of 0.5 \(\mu\)M. We observed that 10 \(\mu\)M Pyr3 suppressed the [Ca\(^{2+}\)]\(\text{e}\) 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\(^{2+}\)]\(\text{e}\), we down-regulated TRPC3 protein expression using siRNA. As expected, down-regulation of TRPC3 with siRNA suppressed the elevation of [Ca\(^{2+}\)]\(\text{e}\), 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 \(\mu\)M), 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-\(\gamma\) (5). In addition, pretreatment of BDNF suppressed the IFN-\(\gamma\)-induced elevation of [Ca\(^{2+}\)]\(\text{e}\), along with a
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-$\gamma$ (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-$\gamma$ 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-$\gamma$-induced intracellular $Ca^{2+}$ elevation, 3 M 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. 5A) microglial cells. Thus, TRPC3 channels could also contribute to the maintenance of IFN-$\gamma$-induced sustained intracellular $Ca^{2+}$ elevation in rodent microglial cells used in this study.

TNF-$\alpha$, one of the proinflammatory cytokines, was shown to induce a gradual increase in intracellular $Ca^{2+}$ in cultured astrocytes at a concentration of 2 $\mu$g/ml (20). In the present study, 2 $\mu$g/ml TNF-$\alpha$ 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 TNF-$\alpha$ until the end of recording. Interestingly, 3 $\mu$M Pyr3 applied after the onset of TNF-$\alpha$-induced intracellular $Ca^{2+}$ elevation did not affect $[Ca^{2+}]_i$ in 6-3 ($n = 21$) and primary ($n = 58$; Fig. 5B) microglial cells. These suggest that TRPC3 channels could not be important for the mainte-
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 \(\mu\)g/ml TNFα induced a gradual increase in DAF-2 fluorescence in both 6-3 (\(n = 101\); Fig. 6A) 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 \(\mu\)M 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 \(\mu\)g/ml) also induced a gradual increase in the DAF-2 fluorescence (Fig. 6B). 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. 6D). In contrast, 24 h pretreatment of both BDNF (20 ng/ml) and Pyr3 (0.2 \(\mu\)M) did not reduce the amplitude of TNFα-induced increase in the DAF-2 fluorescence in 6-3 microglial cells.
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(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+}], 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+}], in rodent microglial cells (data not shown). These suggest that BDNF-induced elevation of basal levels of [Ca^{2+}], 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+}], 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+}], oscillation (24), suggesting that TRPM2 channels might be involved in the TNFα-induced sustained [Ca^{2+}], 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.

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