

# Role of Ciliary Neurotrophic Factor in Microglial Phagocytosis

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**Abstract** Microglia, CNS-resident macrophages, serve as scavengers to remove cellular debris and facilitate tissue remodeling in the developing and injured CNS. Little is known as what and how microenvironmental factors mediate the phagocytotic ability of microglia. Our previous study has indicated that treatment with glial cell line-derived neurotrophic factor (GDNF) increased the phagocytotic activity of primary rat microglia possibly through the upregulation of  $\alpha 5$  integrin. In the present study, ciliary neurotrophic factor (CNTF), which has been reported to be produced by glia, was shown to have stimulatory effect on the phagocytosis of primary rat microglia and mouse microglial cell line BV2.  $Ca^{2+}$  imaging analysis and the application of intracellular calcium chelator BAPTA-AM revealed that CNTF-induced increase in microglial phagocytosis was mediated by a calcium signaling pathway. Furthermore, treatment with CNTF led to an increase in the expression of  $\alpha v$  integrin, which has been reported to be involved in the phagocytosis of the apoptotic cells. In

summary, we have provided evidence that CNTF can increase microglial phagocytosis through a calcium-mediated pathway. Our results also suggest that the upregulation of  $\alpha v$  integrin by CNTF could be involved in the increased phagocytotic activity of microglia.

**Keywords** Microglia · Phagocytosis · CNTF · Calcium ·  $\alpha v$  Integrin

## Introduction

Microglia, a major immune cell population in the CNS, rest in healthy adult CNS and become activated by acute and chronic neurodegenerative diseases [1–3]. In the developing CNS, microglia produce neurotrophic factors to mediate neuronal differentiation and survival [1, 4], and play the role in axonal guidance and synaptogenesis [5, 6]. When the CNS is damaged or infected, resting microglia become activated, migrate toward the lesion site, and increase in number. Although microglial activation plays controversial roles in the CNS tissue repair [1, 7], activated microglia have a major impact on the phagocytotic activities of efficiently removing neural cell fragments and myelin debris [2, 4, 8]. Moreover, these cells are believed to be beneficial to neuronal survival in the damaged CNS via the production of neurotrophic factors [1–4, 9] and the regulation of the production of nonpermissive substrata [10].

CNTF is a neurotrophic cytokine that was initially isolated from rabbit sciatic nerve [11] and found to act as a survival factor for parasympathetic neurons of the chick ciliary ganglia [12]. Later on, CNTF is also known to be produced by glia and play the protective role in many neuronal cell types [13]. In addition, CNTF promotes oligodendrocyte survival, maturation, and myelination [14].

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Since low doses of CNTF given by intracerebral delivery have been shown to protect striatal neurons from degeneration and restore neostriatal function, the molecule is considered as an effective therapeutic factor for Huntington's disease [15]. Although a large body of evidence has indicated the neuroprotective action of CNTF, the *in vivo* and *in vitro* observations have indicated that CNTF acts as the stimulator for astrocyte activation [16, 17]. It has also been shown that the administration of CNTF into the developing CNS resulted in microglial transformation into an activated amoeboid shape [17]. The recent study has also demonstrated that cultured microglia are able to express CNTFR $\alpha$  and its accessory receptors (gp130 and the LIF receptor $\beta$ ) [18]. Moreover, stimulation with CNTF enhances the secretion of the glial cell-line derived neurotrophic factor (GDNF) from microglia, which in turn promotes the survival of the spinal motor neurons [18].

In this study, we examined the effect of CNTF on the regulation of microglial phagocytosis which involves the entry of large particles, typically 1  $\mu\text{m}$  or more, including particles as diverse as inert beads, apoptotic cells, and microbes [19]. The correlation between intracellular Ca<sup>2+</sup> levels and microglial phagocytosis after treatment with CNTF was studied. In addition, the production of integrins, which is known to involve microglial phagocytosis [20], was examined in CNTF-treated microglia. Our study provides evidence that CNTF could improve CNS tissue remodeling due to its positive effect on microglial phagocytosis.

## Experimental Procedure

### Materials

Media and antibiotics were purchased from Invitrogen (Carlsbad, CA). Cell culture ware and Petri dishes were from BD Biosciences (San Jose, CA). Fetal bovine serum (FBS) was the product from HyClone (Logan, UT). Poly-D-lysine (PDL), B4-isolectin, BAPTA-AM (intracellular Ca<sup>2+</sup> chelator), endotoxin lipopolysaccharide (LPS), PKH26 red fluorescent phagocytic cell linker kit, anti- $\alpha$ -tubulin antibody, and cytochalasin D were from Sigma (St. Louis, MO). CNTF and GDNF was the product of R&D (Minneapolis, MN). Nile red fluorescent latex microparticles (1  $\mu\text{m}$  diameter) and Ca<sup>2+</sup>-sensitive dye Fluo-4-AM were purchased from Molecular Probes (Eugene, OR). Rat interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) ELISA kits were purchased from R&D (Minneapolis, MN). Bio-Rad DC protein assay kit was from Bio-Rad (Hercules, CA). Nitrocellulose membrane and ECL solution were from Hybond ECL (Amersham, Piscataway, NJ) and NEN LifeScience (Boston, MA), respectively. Rat anti-CD11b

antibody was the product of AbD Serotec (Raleigh, NC). Mouse anti- $\alpha\text{v}$  integrin and mouse anti- $\beta\text{1}$  integrin were from BD Biosciences and Chemicon (Temecula, CA), respectively. Horseradish peroxidase (HRP)-conjugated or biotinylated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

### Cell Cultures

#### *BV2 Cells*

A mouse microglial cell line, BV2, which has been shown to retain the morphological, phenotypical and functional properties for primary rat microglia [21], was maintained as previously described [22].

#### *Primary Culture*

Primary rat microglial culture was prepared as described previously [23]. In brief, cerebral cortices from neonatal Sprague–Dawley rat brains (P1) were removed and carefully dissected. The tissue was dissociated in 0.0025% trypsin/EDTA and passed through a 70  $\mu\text{m}$  pore nylon mesh. After centrifugation, the cell pellet was resuspended in DMEM/F-12 containing 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. The cells (10<sup>7</sup> cells/flask) were then plated onto PDL-coated 75-cm tissue culture flasks. The medium was renewed every 2–3 days. Eight days later, primary rat microglia were collected using the shake-off method [24]. Primary rat microglia were replated either onto the 96-well tissue culture plates or the 35 mm tissue culture dishes at a density of 2  $\times$  10<sup>4</sup> cells/well or 5  $\times$  10<sup>5</sup> cells/dish, respectively. About 18–24 h later, the cells were treated with rat recombinant CNTF and GDNF in DMEM/ F-12 medium containing N1 serum supplement. In general, 95% of cells in the culture consisted of primary rat microglia with B4-isolectin positive staining, whereas less than 5% of cells were GFAP-immunostained astrocytes in the culture.

### Phagocytosis Analysis

#### *The Labeling Assay of the Phagocytic Cell Linker*

The assay was prepared as described previously [25]. A PKH26 red fluorescent phagocytic cell linker kit which was prepared following the procedure provided by the vendor, was added to the culture and incubated for 3 min followed by the addition of 1% bovine serum albumin for 1 min. Cells were fixed in 2% paraformaldehyde for 10 min and counterstained with 0.1% DAPI for 1 min. Four random fields of cells (approximately 80 cells) were counted using a 40 $\times$  object lens under an epifluorescence microscope

equipped with a cooling digital imaging system. The cells with bright, punctuated fluorescent appearance were referred to as the phagocytic cells. The ratio of phagocytic cells to the total counted cells was defined as the phagocytotic index.

#### *The Uptake Assay of Microparticles*

Microglial phagocytosis was also analyzed using the method as previously described [26, 27]. Primary rat microglia were subcultured onto the 35 mm Petri dishes at the density of  $3 \times 10^5$ /dish. After treatment for 24 h, latex microparticles (1  $\mu$ m diameter Nile red fluorescent latex beads) in the amount of  $6 \times 10^7$  particles/dish were added to the cultures for 30 min, and then were washed 3 times with PBS containing 1% BSA to terminate microglial phagocytosis and remove cell surface-bound microparticles. The cells were removed by adding trypsin/EDTA solution and centrifuged at 500g for 5 min. The cell pellet was resuspended in 1 ml PBS containing 0.1% NaN<sub>3</sub> and 1% serum, and then was subjected to flow cytometry (FACSCalibur™, BD Biosciences) to analyze the fluorescent intensity. Briefly, the cells were resuspended in 1 ml FACS buffer and detected in the FL-2 channel (564–606 nm). The phagocytotic activity is represented by the phagocytotic index that was obtained from the fluorescence intensity.

#### Immunofluorescence

After microglia were exposed to latex microparticles in the amount of  $6 \times 10^7$  particles/dish for 30 min, washed in PBS containing 1% BSA three times, and then fixed in 4% paraformaldehyde for 10 min. The cells were incubated with anti-CD11b (1:200) in PBS containing 5% horse serum overnight, followed by the addition of biotinylated secondary antibody for 1 h and fluorescein-avidin D for 45 min. CD11b immunostaining and microparticles in microglia were visualized by confocal microscopy. Alternatively, after exposure to microparticles, the culture was subjected to nuclei counterstaining in PBS containing 1  $\mu$ g/ml DAPI (Pierce, Rockford, IL) for 2 min. microparticles in DAPI-stained cells were visualized under an epifluorescence microscope equipped with a cooling digital imaging system.

#### TNF- $\alpha$ and IL-1 $\beta$ Assay

Primary rat microglia at a density of  $2 \times 10^4$  cells/well were replated onto 96-well plates. About 24 h after treatment with CNTF, the culture media were collected and centrifuged at 1500 rpm for 10 min to remove floating cells. The supernatant was then used for cytokine ELISA assay. TNF- $\alpha$  and IL-1 $\beta$  were measured using an enzyme

immunoassay kit following the procedure provided by the vendor.

#### Calcium Imaging

Intracellular calcium levels were analyzed as described in detail previously [28]. In brief, primary rat microglia were loaded with 5  $\mu$ M of the Ca<sup>2+</sup>-sensitive dye Fluo-4-AM for 20 min in the dark, and then were washed 3 times with a HEPES-buffered Hank's balanced salt solution (HHBSS) containing (in mM) 137 NaCl, 5.4 KCl, 1.26 CaCl<sub>2</sub>, 0.4 MgSO<sub>4</sub>, 0.64 KH<sub>2</sub>PO<sub>4</sub>, 3 NaHCO<sub>3</sub>, 5.5 glucose, and 20 HEPES (pH 7.4). The culture was mounted on an Olympus IX71 Fluorescence inverted microscope equipped with a 40 $\times$  oil-immersion lens, and perfused with HHBSS (or Ca<sup>2+</sup>-free HHBSS plus 1 mM of EGTA) for 30 s. NT-3, CNTF, and GDNF were added to the culture and time-lapse images were recorded for 5 min at 1 s/frame using a cooled CCD camera with TILL Photonics Imaging System Software (IMAGO-QE; TILL Photonics GmbH, CA). The Ca<sup>2+</sup>-sensitive dye, Fluo-4-AM, was excited by the 488 nm line of a xenon short arc lamp and the emission wavelength was measured at 530 nm. Regions of interest (ROIs, containing 10–15 cells) were selected, and the fluorescence intensity was expressed as  $F/F_0$ ;  $F$  was the fluorescence intensity at any time and  $F_0$  was the averaged fluorescence intensity during the first 30 s of HHBSS perfusion.

#### Western Blotting

Cells were washed twice with PBS after harvest and lysed for 30 min on ice in the lysis buffer containing 1% Triton-X 100, 0.05% Tween-20, 10  $\mu$ g/ml PMSF, 4  $\mu$ g/ml pepstatin A, 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM EDTA, 130  $\mu$ M bestatin, 1.4  $\mu$ M E-64, 1  $\mu$ M leupeptin, 0.3  $\mu$ M aprotinin, and 1 mM sodium orthovanadate, and then centrifuged at 12,000 rpm for 10 min. Protein concentration was determined by a Bio-Rad DC kit. Proteins (10–20  $\mu$ g) were separated by 7.5% SDS-PAGE and transferred to a nitrocellulose membrane, which was then incubated with primary antibodies overnight at 4°C followed by HRP-conjugated secondary antibodies and ECL solution. The antibodies used in this study included mouse anti- $\alpha$ -tubulin antibody (1:5000), mouse anti- $\alpha$ v integrin antibody (1:1000), and mouse anti- $\beta$ 1 integrin antibody (1:2500).

#### Statistical Analysis

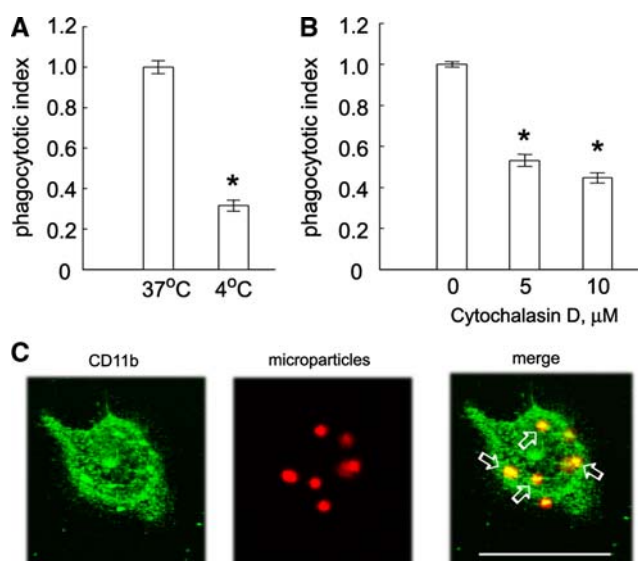
Data are expressed as mean  $\pm$  SEM. Each experiment was repeated at least three times. Statistical significance of differences between the two groups of data ( $P$ -value < 0.05)

was performed using unpaired *t*-test (Minitab, State College, PA).

## Results

### An Increase in the Phagocytotic Activity of Microglia Induced by CNTF

Latex microparticles have been used to study the phagocytotic activity of cultured microglia [26, 29, 30]. To examine the effect of CNTF on microglial phagocytosis, the microglial activity of engulfing microparticles at the size of 1  $\mu\text{m}$  was analyzed after a 24-h treatment. This phagocytotic assay was first validated by examining the inhibition of microglial uptake of microparticles at 4°C after exposure to latex microparticles for 30 min (Fig. 1a). It was also noted that microparticle uptake by microglia was effectively blocked by 5 and 10  $\mu\text{M}$  of cytochalasin D, a potent inhibitor for actin polymerization (Fig. 1b). In addition, confocal microscopy showed that microparticles



**Fig. 1** Phagocytotic assay of microglia by examining engulfing fluorescent microparticles. **(a)** Primary rat microglia were exposed to fluorescent microparticles for 30 min at 37°C or at 4°C. The results showed that 65% inhibition in the microglial phagocytotic activity was induced by incubating the culture at 4°C when compared to the culture at 37°C (100%). **(b)** Treatment with 5 or 10  $\mu\text{M}$  of cytochalasin D caused a 40–55% reduction in the phagocytotic activity of microglia. **(c)** Primary rat microglia were exposed to microparticles (red) for 30 min and then subjected to CD11b (Mac-1) immunostaining (green). Confocal images indicate that microparticles were ingested by a CD11b-stained microglial cell. Data are means  $\pm$  SEM from three experiments and  $*P < 0.05$  was determined by unpaired *t*-test. Scale bar in C, 40  $\mu\text{m}$ . For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article

were internalized in the cytoplasm of microglia stained by the antibody against the surface antigen, CD11b (Fig. 1c).

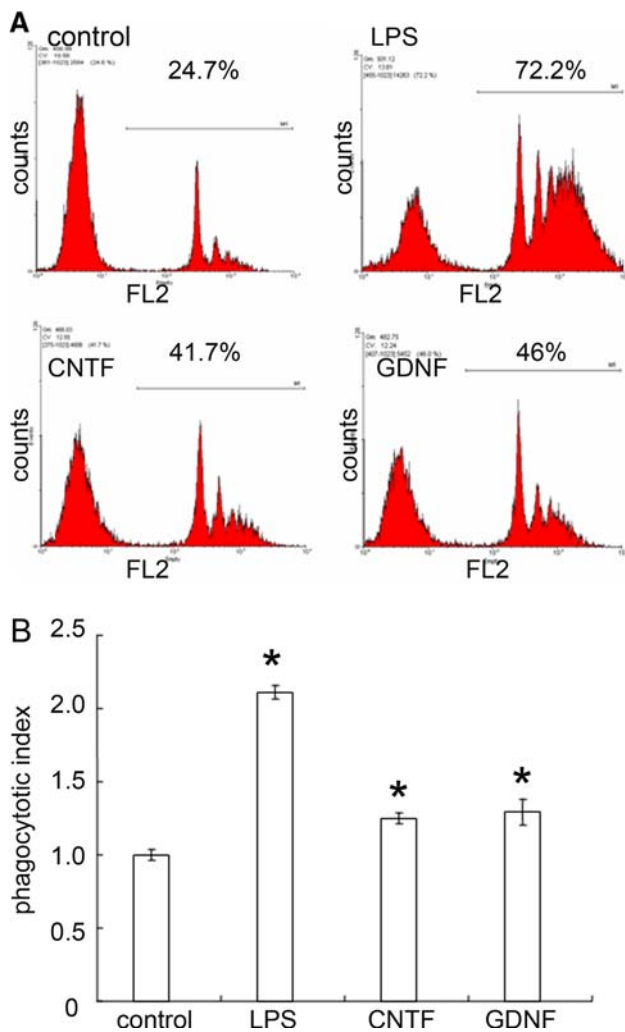
We were first to examine the effect of CNTF on the phagocytotic activity of BV2 cells which are widely used as the in vitro model of microglia. As shown in Fig. 2a, exposure of BV2 cells to CNTF at 10 ng/ml for 24 h caused a 25% increase in the phagocytotic activity of BV2 cells when compared to the control. In agreement with our previous observation using a PKH26 red fluorescent phagocytic cell linker kit, we showed that treatment with the TGF- $\beta$  family member [25], GDNF, improved the ability of BV2 to engulf microparticles (Fig. 2b). Considering there could be discrepancies between the behavior of BV2 cell line and that of primary microglia, the stimulatory effect of CNTF was further confirmed on microglia isolated from neonatal rat cerebral cortices. The phagocytotic activity assay using the PKH26 red fluorescent phagocytic cell linker kit also indicated that the phagocytotic ability of primary rat microglia was improved by treatment with 10 ng/ml of CNTF for 24 h (Fig. 3a). Furthermore, the uptake of microparticles by fluorescence microscopy and flow cytometric analysis indicated that a three-fold increase in the phagocytotic index was induced in primary rat microglia after treatment with 10 and 20 ng/ml of CNTF for 24 h (Fig. 2b, c), although the increase was not dose-dependent. In contrast, treatment with 20 ng/ml of NT-3 did not increase the ingestion of microparticles by microglia (data not shown). These observations point to the specificity of CNTF effect on the microglial phagocytotic activity.

### TNF- $\alpha$ and IL-1 $\beta$ Levels in CNTF-Treated Microglia

Microglia have been reported to become amoeboid in developing brain after injection with CNTF [17]. To test whether CNTF could activate microglia to increase proinflammatory cytokines, the levels of IL-1 $\beta$  and TNF- $\alpha$  in primary rat microglia were analyzed after treatment with CNTF. As shown in Fig. 4, no significant difference in secreted TNF- $\alpha$  and IL-1 $\beta$  levels was observed in the culture of primary microglia treated with CNTF for 24 h. This reveals that CNTF-induced increase in microglial phagocytosis was not resulted from the action of proinflammatory cytokines.

### Increased Expression of $\alpha\text{v}$ Integrin in Microglia by CNTF

Phagocytosis is mediated by complex membrane receptors and molecular mechanism. The  $\beta 1$  integrin regulates the phagocytotic uptake of bacteria by macrophages [31, 32]. The  $\alpha\text{v}$  integrin which heterodimerizes with  $\beta 5$  integrin mediates the binding and internalization of apoptotic cells,



**Fig. 2** Enhanced phagocytotic activity of BV2 cells by CNTF. BV2 cells were treated with CNTF (10 ng/ml) or GDNF (20 ng/ml) for 24 h. They were then incubated with fluorescent microparticles during the last 30 min before harvesting. (a) Representative histogram (A) indicates the phagocytotic activity of BV2 cells treated with CNTF, GDNF and LPS. BV2 cells treated with LPS (10 ng/ml) were used as the positive control for this phagocytotic assay. (b) The results express as the phagocytotic index which is relative to the phagocytotic activity of untreated BV2 cells. Data are means ± SEM from three experiments, \**P* < 0.05 as determined by unpaired *t*-test

subsequently improving phagocytosis of the apoptotic cells [33]. Accordingly, we examined the expression of  $\alpha$ v integrin and  $\beta$ 1 integrin in CNTF-treated microglia. Western blotting showed that treatment with 10 ng/ml of CNTF induced an increase in the production of microglial  $\alpha$ v integrin (Fig. 5a), while the levels of  $\beta$ 1 integrin in microglia was not changed significantly by a 24 h-treatment with CNTF (Fig. 5b). Since GDNF has been previously reported to increase microglial phagocytotic activity, we also investigated whether GDNF had any effect on the production of  $\alpha$ v integrin. As shown in Fig. 5c, 20 ng/ml of GDNF did not show any significant

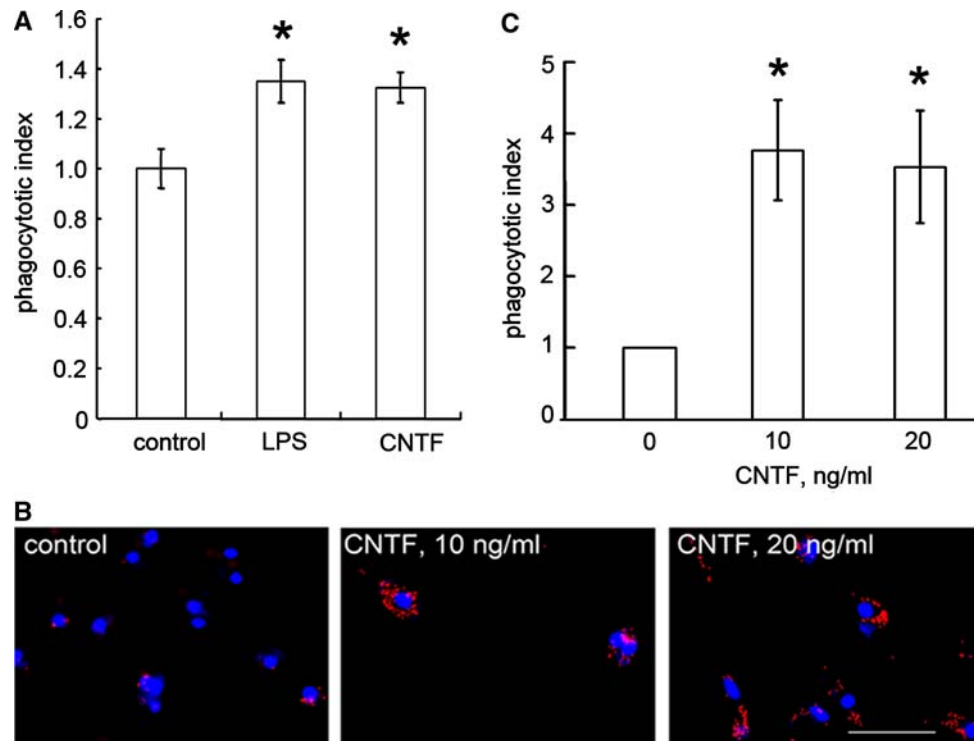
effect on the expression of  $\alpha$ v integrin. We have demonstrated that 20 ng/ml of GDNF increased the production of  $\alpha$ 5 integrin in microglia [25]. However, here we found no effect of CNTF on microglial  $\alpha$ 5 integrin expression (data not shown). These results imply that CNTF and GDNF mediate the expression of different integrins, which in turn regulate microglial phagocytosis.

#### Intracellular Ca<sup>2+</sup> Rise in Primary Rat Microglia by CNTF

Ca<sup>2+</sup> plays the role in the stabilization of integrin clusters [34] and phagosome formation [35]. To test the possibility that CNTF-induced phagocytotic activity of microglia might be mediated by calcium-dependent pathway, we first examined the change in intracellular Ca<sup>2+</sup> levels after the addition of CNTF. As shown in Fig. 6, the application of 10 ng/ml of CNTF to primary rat microglial cultures significantly increased intracellular Ca<sup>2+</sup> levels in a time-dependent manner. Interestingly, GDNF, which was previously found to have the ability to improve microglial phagocytosis [25], also induced a rapid increase in intracellular Ca<sup>2+</sup> levels in microglia. However, NT-3, which was shown to be of no effect on microglial phagocytosis [22], did not cause a rise in the intracellular Ca<sup>2+</sup> levels (Fig. 6). The negative finding supports the positive observations that Ca<sup>2+</sup> signaling could play the role in the regulation of CNTF- or GDNF-treated microglia to take up microparticles. Indeed, further experiments indicated that CNTF failed to increase the phagocytotic activity of microglia after pretreatment with intracellular Ca<sup>2+</sup> chelator, BAPTA-AM (Fig. 7a). We next investigated whether the rise of intracellular Ca<sup>2+</sup> by CNTF plays the role in the regulation of microglial  $\alpha$ v integrin production. We found that pretreatment with BAPTA-AM blocked the CNTF effect on the upregulation of  $\alpha$ v integrin expression in microglia, indicating that the expression of microglial  $\alpha$ v integrin was mediated by the CNTF-induced increase of intracellular Ca<sup>2+</sup> (Fig. 7b).

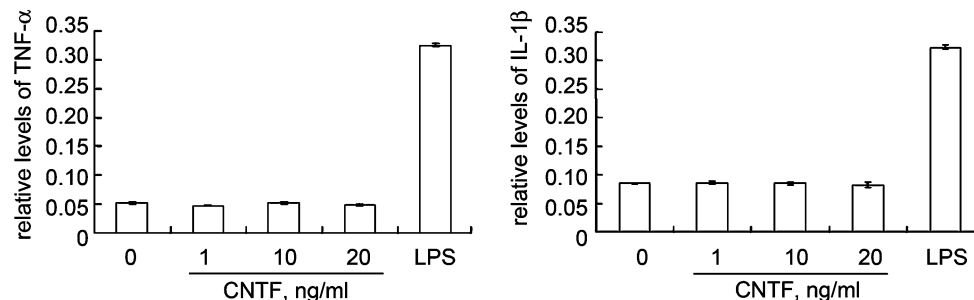
#### Discussion

The physiological role of neurotrophic factors produced by neurons and activated glia in microglial function is not well understood. Previously we showed that NT-3 played as an anti-inflammatory factor for LPS-activated microglia [22] and that GDNF enhanced microglial phagocytosis [25]. Considering the therapeutic effect of CNTF on neurological disorders such as human Huntington’s disease [15, 36], the present study was to investigate whether CNTF had a regulatory role in microglial phagocytosis. Examination of the phagocytotic activity of BV2 cells and primary rat microglia revealed a



**Fig. 3** Increased phagocytotic activity of primary rat microglia by CNTF. **(a)** Primary rat microglia were treated with 10 ng/ml of CNTF for 24 h and then incubated with a phagocytic cell linker kit for 3 min as described in section “Materials and Methods”. Primary rat microglia treated with LPS (10 ng/ml) were used as the positive control for this phagocytotic assay. **(b and c)** Primary rat microglia were treated with fluorescent microparticles for 30 min and were subjected to flow cytometric analysis as described above. Data in **a**

and **c** are expressed as phagocytotic index which is relative to the phagocytotic activity of microglia without CNTF treatment. Images shown in **b** indicate microparticles (red) taken up by microglia (DAPI nuclear staining, blue) treated without or with CNTF (10 and 20 ng/ml). Values are means  $\pm$  SEM from three experiments,  $*P < 0.05$  as determined by unpaired *t*-test. Scale bar in **b**, 50  $\mu$ m. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article



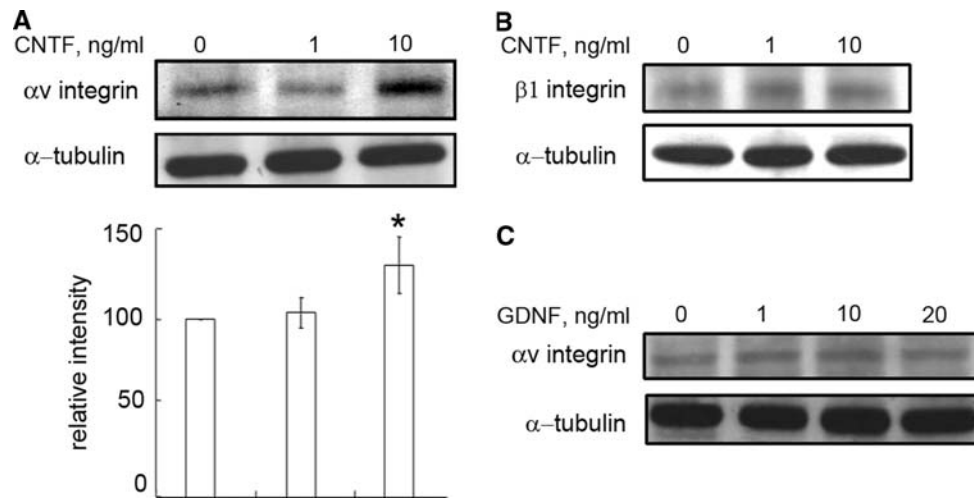
**Fig. 4** CNTF effect on the levels of TNF- $\alpha$  and IL-1 $\beta$  in the culture medium of primary rat microglia. Microglia were treated with CNTF at the distinct concentrations as indicated above. The culture media were collected at 24 h, and subjected to TNF- $\alpha$  and IL-1 $\beta$  ELISA

assays. The culture treated with 10 ng/ml of LPS was referred as a positive control for the assay of TNF- $\alpha$  and IL-1 $\beta$  levels. Values were determined using mean  $\pm$  SEM

stimulatory effect of CNTF on microglial phagocytosis. In response to CNTF, microglia increased the expression of  $\alpha$ v integrin, which was shown to mediate the phagocytosis of apoptotic cells by dendritic cells [33]. The addition of CNTF caused a slow increase in the levels of intracellular  $Ca^{2+}$  in microglia, implying the involvement of  $Ca^{2+}$  signaling in CNTF-mediated microglial phagocytosis. The suggestion is supported by the observations that intracellular  $Ca^{2+}$

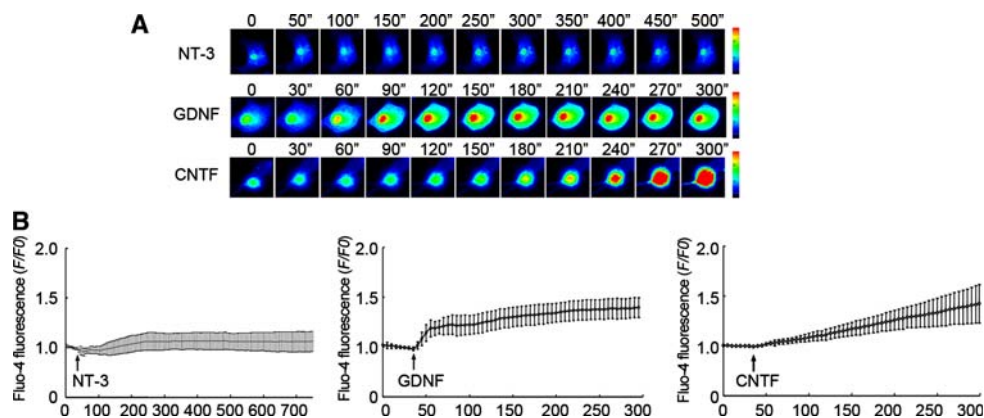
chelator, BAPTA-AM, significantly abrogated the CNTF effect on the production of  $\alpha$ v integrin in microglia and microglial phagocytosis.

Recent studies from Furukawa and his coworkers using macrophages isolated from the heterozygous (+/–) GDNF gene-deficient mice or wild-type (+/+) mice have indicated that GDNF enhanced phagocytic activity of macrophages [37]. In the past, we used the PKH26 red fluorescent dye



**Fig. 5** Increased levels of  $\alpha v$  integrin in microglia after a 24-h treatment with CNTF. Primary rat microglia were treated for 24 h with 1 and 10 ng/ml of CNTF (a, b). In another experiment, microglia were treated with GDNF at the different concentrations as indicated above (c). Cell extracts were analyzed by Western blotting for the levels of  $\alpha v$  integrin (a, b) and  $\beta 1$  integrin (b). Data are representative

of three similar experiments. Relative levels of  $\alpha v$  integrin to  $\alpha$ -tubulin in microglia with treatments were normalized to that measured in the control. Values were determined using mean  $\pm$  SEM of three experiments. \* $P < 0.05$  (unpaired  $t$ -test) compared to the control

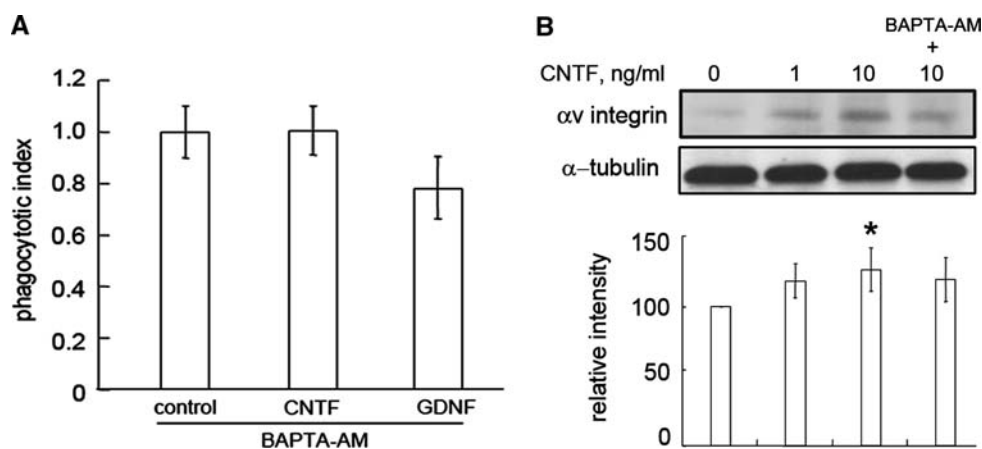


**Fig. 6** Increase of intracellular  $Ca^{2+}$  rise in primary rat microglia by CNTF and GDNF. To examine intracellular  $Ca^{2+}$  change, microglia were preloaded with Fluo-4-AM (5  $\mu$ M) for 20 min, and then perfused in HHBSS containing NT-3 (20 ng/ml), GDNF (20 ng/ml) or CNTF (10 ng/ml) at the different concentrations as indicated above. Intracellular  $Ca^{2+}$  levels were analyzed as described in section “Materials and Methods”. (a) The series of  $Ca^{2+}$  images were recorded from the cultures treated with NT-3, GDNF and CNTF. (b)

The time-course tracing of the relative fluorescence intensity  $F/F_0$  indicated that intracellular  $Ca^{2+}$  levels were increased after the addition of CNTF and GDNF (arrows). It was noted that no change in intracellular  $Ca^{2+}$  levels in the control (vehicle) was observed. Data represent means ( $\pm$ SEM) from a total of 10–15 cells in one representative experiment. Within each optic field, the percent of cells that responded to the treatment was 95%. A total of three independent experiments were performed and gave similar results

for the phagocytosis assay of GDNF-treated microglia form aggregates or particulates that can be uptaken only by phagocytotic cells [25]. Similar to the previous observations, we showed here that GDNF effectively improved microglial ability to engulf microparticles, which further confirmed the role of GDNF in microglial phagocytosis. By using two phagocytosis assays, we also demonstrated that the application of CNTF to microglia enhanced their phagocytotic activity. Recent report from Chan et al. has indicated that  $TNF-\alpha$  triggers an oxidant-dependent

mechanism to activate Rho and causes the morphologic changes of macrophages, which subsequently decreases macrophage clearance of apoptotic cells [38]. No change in  $TNF-\alpha$  production and oxidant generation was observed in microglia after treatment with CNTF, which showed that  $TNF-\alpha$ /oxidant effect is not involved in the mechanism for CNTF-induced microglial phagocytosis. On the other hand, we have observed a slow rise in intracellular  $Ca^{2+}$  in microglia after the addition of GDNF and CNTF. Although there was only a 25% increase, such an increase implied



**Fig. 7** Blockage of CNTF-induced microglial phagocytosis and  $\alpha v$  integrin production by intracellular  $\text{Ca}^{2+}$  chelator BAPTA-AM. **(a)** Microglia were pretreated with BAPTA-AM (10  $\mu\text{M}$ ) for 30 min, and then stimulated with CNTF (10 ng/ml) and GDNF (20 ng/ml) for 24 h. After exposure to fluorescent microparticles for 30 min, the cells were subjected to the flow cytometric analysis as described in

Materials and Methods. **(b)** Microglia were pre-treated with BAPTA-AM (10  $\mu\text{M}$ ) for 30 min followed by treated with 10 ng/ml of CNTF for 24 h. Relative levels of  $\alpha v$  integrin to  $\alpha$ -tubulin in microglia with treatments were normalized to that measured in the control. Values were determined using mean  $\pm$  SEM of three experiments. \* $P < 0.05$  (unpaired *t*-test) compared to the control

that  $\text{Ca}^{2+}$  signaling might mediate GDNF- or CNTF-induced microglial phagocytosis. This is supported by the observation that neither CNTF nor GDNF can induce an increase in microglial phagocytosis in the presence of the intracellular  $\text{Ca}^{2+}$  chelator. Our results indicated that an increase in microglial phagocytosis induced by CNTF was observed 24 h later, which showed that microglial phagocytosis could be indirectly mediated by CNTF-induced  $\text{Ca}^{2+}$  signaling.

Integrin family members such as  $\alpha 5\beta 1$  and  $\alpha v\beta 5$  are known to be involved in microglial migration, adhesion and phagocytosis [20, 33, 39]. We have corroborated that CNTF-induced increase in microglial phagocytosis is correlated with the upregulation of  $\alpha v$  integrin production in microglia. Unlike our previous study which showed that GDNF might upregulate the production of  $\alpha 5$  integrin to increase microglial phagocytosis [25], we found CNTF had no effect on the expression of microglial  $\alpha 5$  integrin. Similar to GDNF, CNTF was also observed to have no effect on the expression of microglial  $\beta 1$  integrin. Based on the present results, we could not conclusively determine the precise role of  $\alpha v$  integrin in CNTF-mediated microglial phagocytosis. However, the presence of the intracellular  $\text{Ca}^{2+}$  chelator not only blockaded the CNTF-induced microglial phagocytosis, it also effectively reduced the CNTF effect on the expression of microglial  $\alpha v$  integrin. This implies that the  $\text{Ca}^{2+}$  signaling may mediate CNTF-induced production of  $\alpha v$  integrin in microglia to regulate microglial phagocytotic activity.

In conclusion, CNTF may serve as a stimulatory molecule for the microglial phagocytotic activity through  $\text{Ca}^{2+}$ -mediated upregulation of  $\alpha v$  integrin. This study has also

provided evidence that CNTF has a modulatory role in microglial behavior.

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