神經保護之研究 探討神經滋養訊息調控微膠細胞活性

計畫類別：整合型計畫
計畫編號：
執行期間：93年08月01日至94年07月31日
執行單位：國立成功大學生物學系（所）

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報告類型：精簡報告
處理方式：本計畫可公開查詢

中華民國94年05月30日
Potential link between systemic inflammation and neuronal neuroprotection/degeneration—the role of neuron-glia cross talk --
Neuroprotective study: neurotrophic signals modulating microglial activities

行政院國家科學委員會補助專題研究計畫 □成果報告 □期中進度報告

（計畫名稱）

計畫類別：□個別型計畫 √整合型計畫
計畫編號：NSC 93－2321－B－006－010－
執行期間：93年08月01日至94年07月31日

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成果報告類型(依經費核定清單規定繳交)：√精簡報告 □完整報告

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執行單位：國立成功大學生命科學系
中文摘要
關鍵詞：微膠細胞、前發炎細胞激素、神經滋養因子、神經元細胞、神經保護作用

當中樞神經系統受損會造成微膠細胞(microglia)的活化；活化的微膠細胞會釋放神經毒性物質，包括一氧化氮、超氧化物陰離子、前發炎細胞激素(TNF-α及IL-1β)等，阻礙了中樞神經系統的再生；這些神經毒性物質能誘導神經退化性疾病如腦部缺氧、阿茲海默症(AD)、腦部發炎、漢丁頓疾病，脊髓損傷及愛滋病的神經病理現象發生。基於此，抑制微膠細胞的活化是增進中樞系統修復的重要策略。神經滋養因子可有效調節神經細胞的存活、神經分化、及神經可塑性。擁有神經滋養因子接受器的神經細胞及膠質細胞同時也會製造神經滋養因子；許多研究成果都指出，在中樞神經系統受損的情況下，神經滋養因子(BDNF, NT3, NT4, NGF, GDNF及CNTF)能保護多種類型的神經細胞，表示這些神經滋養因子具有臨床應用的價值。

然而，神經滋養因子是如何調控微膠細胞的活化仍是未知的。在本實驗室先前研究指出在脂多醣(LPS)存在的情況下，以神經滋養因子-3前處理微膠細胞的細胞株－BV2，會降低一氧化氮及前發炎細胞激素(TNF-α及IL-1β)的產生。神經滋養因子-3所調控的訊息傳遞路徑除了致裂原活化蛋白酶(MAPK)及磷脂肌醇-3激酶(PI3-kinase)，且抑制NF κB的活化。此外，二維膠體電泳分析結果顯示有八個以上的蛋白質表現量受神經滋養因子-3前處理所調節。再者，前處理神經滋養因子-3於微膠細胞，似乎降低脂多醣所引起的吞噬作用能力；但是，以神經膠質細胞神經營養因子(GDNF)處理微膠細胞卻可增加其吞噬作用的能力。另一方面，本子計畫同時並探討睫狀神經滋養因子(CNTF)對於微膠細胞的活化作用。結果顯示，前處理睫狀神經滋養因子(CNTF)24小時後再處置脂多醣後其iNOS的表現，而前處理睫状神經滋養因子24小時後再有脂多醣神經滋養因子存在下後處理脂多醣於微膠細胞，卻增加誘導性一氧化氮合成酶(iNOS)的表現。

綜合以上實驗結果可知神經滋養因子經由不同的分子機制調控微膠細胞活性。

英文摘要
Key words: microglia, neurons, neuroprotection, neurotrophic factors, proinflammatory cytokines

Microglial activation occurs after injuries to the central nervous system (CNS), causing the difficulties of CNS regeneration. Activated microglia generates neurotoxins including nitric oxide (NO), superoxide (O2·−), proinflammatory cytokines (TNF-α and IL-1β), and they are believed to have the critical roles in neuropathogenesis of neurodegenerative diseases, such as cerebral ischemia, Alzheimer's disease (AD), brain inflammation, Huntington's disease, spinal cord injury, and AIDS. Thus, suppression of microglial activation is an important strategy to improve CNS repair. Neurotrophins (NTs) are potent regulators for survival, neural differentiation, and neuroplasticity. Neurons and glial cells that express their receptors can also produce NTs. A large body of evidence indicates that NTs (BDNF, NT3, NT4, NGF, GDNF, and CNTF) protect many neuronal populations from various injuries, indicating that these NTs hold promise for clinic application. However, little is known about NT signals on the regulation of microglia activities. Our previous studies showed that pretreatment with NT3 (preNT-3) reduces the production of NO and proinflammatory cytokines produced by LPS-activated microglia. In addition to the involvement of MAPK and PI3 K signaling pathways in preNT-3 action on the inhibition of microglial activities, NF κB activation in LPS-activated microglia was inhibited by preNT-3. Furthermore, 2D-PAGE revealed up to 8 regulated proteins by NT-3 pretreatment. Moreover, it seems like that preNT-3 reduced the phagocytic ability of LPS-activated microglia; however, treatment of microglia with GDNF increased their phagocytosis. In the first year of the grant period, we also examined the effect of CNTF on microglial activities. The result indicated that pretreatment of LPS-activated microglia with CNTF for 24 h reduced microglial iNOS production, whereas CNTF pretreatment for 24 h and continuous CNTF treatment in the presence of LPS increased the production of iNOS in
LPS-activated microglia. Based on the findings, we conclude that distinct neurotrophic factors induce different molecular mechanisms to mediate microglial activities.

**Introduction**

Microglia, CNS resident macrophages (Streit, 1995; Streit et al., 1999), are quiescent in the normal CNS, and they are activated by cytokines produced by infiltrating immune effectors after CNS injury (Gonzalez-Scarano and Baltuch, 1999; Stoll and Jander, 1999; Liu and Hong, 2003). Activated microglial cells are observed in the injured CNS, and they are believed to have the regulatory role in CNS pathophysiology, including cerebral ischemia, brain inflammation (the project 4), Alzheimer’s disease (the project 3 and project 4), Huntington’s disease (the project 2 and project 3), spinal cord injury, and AIDS (Kreutzberg, 1996; Stoll and Jander, 1999; Nakajima and Kohsaka, 2001). Morphological examination indicates that microglia undergo dramatic transformation from resting ramified to activated amoeboid form (Kreutzberg, 1996). Activated microglia primarily produce proinflammatory cytokines including IL-1β and tumor necrosis factor-α (TNF-α), and generate nitric oxide (NO), reactive oxygen species (ROS) such as superoxide anion (O₂⁻), fatty acid metabolites such as eicosanoids, quinolinic acid and hydrogen peroxide. NO can rapidly react with O₂⁻ to produce peroxynitrite anion (Banati et al., 1993; Brosnan et al., 1994; Liu and Hong, 2003). A growing body of evidence indicates that excessive expression of activated microglia secreted factors described as above is deleterious to neurons (Boje and Arora, 1992; Chao et al., 1992; McGuire et al., 2001). Moreover, the synthesis of the inducible form of NO synthase (iNOS) along with the release of NO by microglia is correlated with the progression of neurodegeneration, both in vivo and in vitro (Boje and Arora, 1992; Chao et al., 1992; Espey et al., 1997). Accordingly, it is believed that the reduction in microglial generated ROS and proinflammatory cytokines after CNS injury can suppress neuronal cell death after CNS injury.

On the other hand, microglia phagocytose degenerating cellular fragments, showing they are needed for tissue/system modeling during development or after CNS injury (Chan et al., 2001). In addition to being debris scavengers, activated microglia can secrete neurotrophic factors (NTs), growth factors, indicating their beneficial role in CNS repair (Streit, 2002). Evidence has shown that vigorous cell death happens in the developing CNS (Oppenheim, 1991). Yet, the tissue damage during normal CNS development is not observed, implying that the harmful inflammatory response may not happen at that stage. Perhaps, there may be protective factors in the developmental environment, which can modulate microglial activation and suppress the tissue damages.

The NTs have numerous important functions in the development and maintenance of both the CNS and PNS (Patapoutian and Reichardt, 2001). In the CNS and PNS, NTs act as survival and differentiation factors (Huang and Reichardt, 2001). Among NTs, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) are derived from a common ancestral gene. They are similar in sequence and structure (Huang and Reichardt, 2001). Their actions are through the activation of the NT receptors (Trks), transmembrane tyrosine kinase receptors (NGF binding to TrkA. BDNF and NT-4 to TrkB. NT-3 predominantly to TrkC). Activation of tyrosine kinase–mediated signaling by Trk receptors is well known to promote survival and/or differentiation of many neuronal populations. Ciliary neurotrophic factor (CNTF) and glial cell line derived neurotrophic factor (GDNF) are derived from the distinct gene family. CNTF through its receptor system, CNTFRα and LIFβ, exerts the survival effect on developing and injured motoneurons (Hunag and Reichardt, 2001). GDNF acts at GFRα and c-Ret to induce neuroprotection for many neuronal populations (Airaksinen and Saarma, 2002).

The observations showing that activated microglia and astrocytes (the project 1 and project 2) produce various NTs have been reported, implying that activated glia may contribute to
neuroprotection and tissue regeneration. GDNF, CNTF, NT3, NT4/5, NGF, and BDNF can be produced in neurons, glia or both. Their receptors also present on neurons and glia. Many studies and evidence have shown that NTs rescue neuronal cell survival in vivo and in vitro by directly acting on neurons to protect them from insults (Thoenen and Sendtner, 2002). Thus, NTs are thought to have the potential therapeutic function for many neurological disorders (Coumans et al., 2001; Sayer et al., 2002). However, it is poorly understood if NTs protect neurons via an indirect pathways inducing that NTs reduce the production of harmful inflammatory agents by acting on activated microglia.

The majority of NTs function is focused on neuronal survival, neuronal differentiation and neuronal function. Elkabes et al (1996), however, have indicated that microglia can proliferate in response to NTs. It has been suggested that NTs produced by microglia can act on microglia via autocrine and/or paracrine fashion (Nakajima et al., 1998). Moreover, NTs can increase microglial acid phosphatase activity and secretion of plasminogen and urokinase type-plasminogen activator (Nakajima et al., 1998). NGF, BDNF, and NT-3 have been also shown to inhibit microglial MHC class II expression and costimulatory molecules (Neumann et al., 1998; Wei and Jonakait, 1999). In addition, NT3 has been found to suppress lipopolysaccharide (LPS)-stimulated NO production in microglia (Tseng and Huang, 2003). Nevertheless, the biological functions of NTs on microglia are not fully understood.

**Specific aims**
1. To determine actions of neurotrophic factors (NTs) on microglial activation: iNOS and phagocytosis.
2. To understand molecular mechanisms for NT-mediated microglial activities.

**Materials and methods**

**Primary rat microglial preparation**
Cerebral cortices from neonatal Sprague–Dawley rat brains (P1) were removed and carefully dissected. The tissue was dissociated in papsin and passed it through a 70 μm-pore nylon mesh. After centrifuge, the cell pellet was resuspended with Dulbecco’s modified Eagle’s medium (DMEM)/ F-12 (1:1) containing 10% heat inactivated FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. The cells at a density of 10^7 cells/ml were then plated onto poly-D-lysine (Sigma) coated 75-cm culture flasks. The culture medium was renewed every 2 d. 8 d later, microglia were collected using the shake-off method. The culture purity was determined by staining for microglial cellular marker B4 isolectin (Sigma).

**Western blot analysis.** Protein samples were extracted by gently homogenizing cells or tissues on ice using PBS containing 1% SDS, 1 mM phenylmethyl-sulfonfluoride (PMSF), 1 mM EDTA, 1.5 mM pepstatin, 2 mM leupeptin, and 0.7 mM aprotinin. Protein concentration was determined using Bio-Rad DC kit. 10-20 μg of total proteins will be loaded onto 7.5% SDS-PAGE, and then transferred to nitrocellulose membrane. The protein was identified by incubating the membrane with specific antibodies overnight at 4°C, followed by horseradish peroxidase conjugated secondary antibodies and ECL solution.

**Immunofluorescence.** After primary microglia were pretreated with 20 ng/ml of NT-3 for 24 h followed by 10 ng/ml of LPS stimulation for 24 h, the cultures were fixed in 4% paraformaldehyde for 10 min, and then incubated with 0.1% Triton X-100 in PBS for 30 min followed by rabbit anti-iNOS antibodies (1:200; Calbiochem) in PBS containing 5% horse serum overnight. The cultures were then incubated with biotinylated secondary antibodies (Vector, Burlingame, CA) for 1 h, and fluorescein-avidin D (Vector) for 45 min, followed by nuclei counterstaining in PBS containing 1 μg/ml of using 4’, 6-diamidine-2’-phenylindole, dihydrochloride (DAPI; Pierce, Rockford, IL) for 5 min. Alternatively, the cultures were incubated in PBS containing biotinylated B4 isolectin (1:200; Sigma) overnight at 4°C and
Rhodamine-avidin D (1:200; Vector) for 1 h at RT, followed by DAPI counterstaining for 5 min. The slides were then mounted, and the results were observed under a fluorescence microscope (Nikon, Japan).

**Phagocytosis assay.** Rat primary microglia at the density of 5 x 10^4 cells/well were replated onto a 24-well plate, and then treated with 20 ng/ml of NT-3 for 24 h followed by stimulation with 10 ng/ml of LPS for 24 h. PKH26 red fluorescent phagocytic cell linker kit (Sigma) 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 the counterstained with 0.1% DAPI for 1 min. Four random fields of cells were counted using a 40x object lens under epifluorescence microscope equipped with a cooling digital imaging system (Photometrics CoolSNAP, Roper Scientific, Inc, Japan). The cells with bright punctuate fluorescent appearance were referred as phagocytic cells. The ratio of phagocytic cells to total counted cells is defined as phagocytotic index.

**Nuclear protein extraction and electrophoretic mobility shift assay (EMSA).** BV2 cells (5 x 10^5 cells/dish) were replated onto 60 mm culture Petri-dishes, and then treated with 20 ng/ml of NT-3 for 24 h followed by stimulation with 10 ng/ml of LPS for 6 h. Nuclear proteins were extracted using the NE-PER kit by cell lysis and centrifugation according to the procedure of the manufacturer (Pierce). NFκB double-stranded DNA oligonucleotides (Promega) was end-labeled with [γ-32P] using the T4 polynucleotide kinase (Promega) at 37°C for 10 min, and purified by passage through G25 spin column. Twenty microgram of nuclear protein extracts, binding buffer (10 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, and 5% glycerol), and 1 µg poly dIdC in a final volume of 20 µl were incubated in the presence of 5 ng of end-labeled oligonucleotide at room temperature for 20 min. To determine the specificity of NF-κB-p65 DNA binding, the nuclear extract from LPS-activated BV2 cells were incubated with 5 ng of end-labeled oligonucleotide containing binding buffer without or with 1 µl of anti-NFκB-p65 antibodies (Cell Signaling) at RT for 20 min. The reaction mixture was fractionated on a 6% polyacrylamide gel (2 h, 180 V). The gel was dried and exposed to film at -70°C for 1-2 h.

**Results**

**Effect of NT-3 on microglial activities**

The primary microglia were exposed to NT-3 at 1, 10 and 20 ng/ml for 24 h before the cells were stimulated for 24 h with LPS at 10 ng/ml in the continuous presence of NT-3. The results showed that preNT-3 significantly reduced the expression of iNOS proteins (Fig. 1A) and NO production (Fig. 1C) in primary microglia activated by LPS along with a dose-dependent pattern. Examination with immunofluorescence staining of primary microglia indicated that substantial increase in iNOS immunostaining was observed in LPS-activated cultures (Fig. 2D), whereas the iNOS immunostaining was reduced in LPS-activated cultures with preNT-3 (Fig. 2F). Morphological investigation also indicated that preNT-3 LPS-stimulated microglia were transformed to a less activated state (Fig. 2F, arrows), when compared to that seen in LPS-treated control (Fig. 2D, arrows).

We also examined the production of TNF-α and IL-1β in primary microglia with preNT-3. We found that the levels of both TNF-α and IL-1β production in primary microglial cultures, as monitored by ELISA, were significantly decreased when the cultures were pre-treated with NT-3 for 24 h prior to LPS stimulation (Fig. 3). Furthermore, investigation of microglial phagocytosis indicated that preNT-3 resulted in significantly reduced phagocytosis in LPS-activated BV2 cells (Fig. 4B), and induced a decreased trend in the phagocytic ability of primary microglia (Fig. 4A)

We exposed BV2 cells to NT-3 in the presence of MAP kinase inhibitor U0126 or PI3 kinase inhibitors LY 294002 for 2 h followed by 22h in the absence of these inhibitors before LPS stimulation. The effect of these inhibitors on the production of iNOS in LPS-stimulated BV2
cells was examined by western blot analysis. As shown in Fig. 5, the inhibitory effect of preNT-3 on the production of iNOS in LPS-stimulated BV2 was blocked by either U0126 or LY294002, suggesting that activation of both pathways is required for the NT-3 effect. One the other hand, western blot analysis showed that the level of nuclear NFκB-p65 subunit was found to be significantly reduced by preNT-3 in LPS-stimulated BV2 cells (Fig. 6A). Examination of NFκB activity also indicated that preNT-3 extensively inhibited LPS-induced NFκB-p65 DNA binding (Fig. 6B). Treatment of nuclear extract with anti-p65 antibodies was performed to elucidate the nature of the NFκB-p65 DNA binding complex activated by LPS (Fig. 6C).

In addition, the results from 2D-PAGE analysis indicated up to 8 regulated proteins found in LPS-activated microglia with preNT-3 when compared to that observed in the control.

**Microglial phagocytosis regulated by GDNF**

GDNF has recently been reported to enhance the phagocytotic activity of macrophage. Since microglia exhibit typical macrophage behaviors, such as phagocytosis, the effect of GDNF on microglial phagocytotic ability was examined in this study. We found that the phagocytotic uptake of primary rat microglia was increased in response to GDNF (Fig. 8).

**Effect of CNTF on the production of iNOS in LPS-activated microglia**

We found that pretreatment with CNTF for 24 h caused a downregulation of iNOS production in LPS-activated microglia (Fig. 9). Yet, pretreatment of microglia with CNTF for 24 h followed by an additional CNTF for 24 h in the presence of LPS synergistically increased iNOS production in microglia. This indicates that CNTF exerted dual effects on the regulation of microglial activities.

**Conclusion**

1. NT-3 pretreatment mediates microglial activities through MAPK and PI3K signaling pathways and the reduction of NFκB activation.
2. In contrast to NT-3 pretreatment, treatment of microglia with GDNF can increase their phagocytosis.
3. CNTF mediates the production of iNOS in activated microglia in the positive and negative manner.

**References**


Publication list
Pretreatment with NT-3 reduces the production of iNOS and NO in primary microglia activated by LPS. Primary microglia were pre-treated for 24 h with NT-3 (preNT-3) followed by 10 ng/ml of LPS stimulation for 24 h in the continuous presence of NT-3 (A,C). Alternatively, primary microglia were cotreated for 24 h with 10 ng/ml of LPS and NT-3 (coNT-3) at distinct concentrations indicated as above (B, D). Levels of nitrate and nitrite in the cell supernatant of primary microglia were measured using a colorimetric detection of nitrite as an azo dye product of the Griess Reaction (C, D). Effect of PreNT-3 at 1, 10 and 20 ng/ml on the cell viability of primary microglia activated by 10 ng/ml of LPS was studied by WST-1 cell viability assay (E). Data are the means of values in triplicate (± S.D.). *p <0.01 compared with the LPS-activated control.

Immunofluorescence examination for iNOS in LPS-activated microglia with or without preNT-3. Primary microglia were treated with 20 ng/ml of NT-3 for 24 h followed by 10 ng/ml of LPS stimulation for 24 h, and then subjected to immunofluorescence of iNOS (red; B,D,F,H) or B4 isoclectin (red; A,C,E,G), followed by nuclei counterstain using DAPI (blue). Scale bar, 50 µm.

Pretreatment with NT-3 reduces the level of TNF-α and IL-1β in primary microglia activated by LPS. Primary microglia were treated with preNT-3 at distinct concentrations indicated as above, followed by 10 ng/ml of LPS stimulation for 24 h in the continuous presence of NT-3. Levels of TNF-α and IL-1β in the cell supernatant were measured using ELISA kits. Data are the means of values in triplicate (± S.D.). *p <0.01 compared with the LPS-activated control.
Figure 4. Effect of preNT-3 on the phagocytotic ability of microglia. Primary microglia (A) and BV2 cells (B) were treated with 20 ng/ml of preNT-3 for 24 h followed by 10 ng/ml of LPS, and then subjected to phagocytosis analysis. Values were determined using mean ± S.D. of three experiments. *p < 0.05 compared with the control. #p<0.05 compared with the LPS-activated group. Scale bar in A, 50 µm.

Figure 5. Inhibitors of MAP kinase and PI3 kinase suppressed the effect of preNT-3 on the production of iNOS in LPS-activated BV2. U0126 (5 µM) and LY294002 (5 µM), MAP kinase and PI3 kinase inhibitors, respectively, were added to cultures 5 min before 20 ng/ml of preNT-3 as described in the materials and methods. Western blot analysis image shown in A-C are representative of three independent experiments. Relative levels of iNOS to α-tubulin in LPS-activated microglia with preNT-3 were normalized to that in LPS-activated microglia without preNT-3. Data are the means of values from three experiments (+ SD). *p < 0.01 compared to the LPS-stimulated control.

Figure 6. Pretreatment with NT-3 reduces the nuclear level of NFκB-p65 and NFκB DNA binding activity in BV2 cells. (A). BV2 cells were treated with 20 ng/ml of preNT-3 for 24 h, and then continuously stimulated by 10 ng/ml LPS for 2 h. The nuclear proteins were isolated and then subjected to western blot analysis for p65 detection. Data are the means of values from three experiments (+ SD). *p<0.01 compared to the LPS-activated control. (B). Nuclear extracts were prepared from BV2 cells after 20 ng/ml of preNT-3 for 24 h and 10 ng/ml of LPS for another 6 h, and then subjected to EMSA. Arrow indicates NFκB-DNA complexes including p65/p50 and p50/p50. NS, nonspecific binding; FP, free probe.
Figure 7. 2D-PAGE analysis. Primary microglia were treated with 20 ng/ml of preNT-3 for 24 h followed by 10 ng/ml of LPS, and then subjected to 2D-PAGE analysis.

Figure 8. Microglial phagocytotic activity improved by GDNF. Primary rat microglia were treated with 20 ng/ml of GDNF or 10 ng/ml of LPS for 24 h, and then incubated with a phagocytic cell linker kit for 3 min as described in Materials and Methods. Three independent experiments were done with similar results, and each experiment was performed in duplicate. Values were determined using mean ± S.D. of three experiments. * $p < 0.05$ (unpaired t test) compared to control. Immunofluorescent images (400x) are representative of three independent experiments.

Figure 9. Microglia were pretreated with 10 ng/ml of CNTF for 24 h, and continuously stimulated with 10 ng/ml of LPS in the presence or absence of 10 ng/ml of CNTF for 24 h.