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FINAL Research Progress Report for

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Anti-inflammatory Effects of Ciliary Neurotrophic Factor (CNTF) on Microglia (05-2913-SCR-E-0)

Term: 6/15/2006 to 6/15/2008 Report date: Aug. 1st 2008

Original Specific Aims:

Aim 1. To examine whether mouse microglia express functional Ciliary Neurotrophic Factor (CNTF) receptor alpha.

Aim 2. To investigate whether CNTF decreases microglial antigen presentation.

Aim 3. To explore whether CNTF decreases microglial myelin phagocytosis.

Project Successes.

During the past two years, a manuscript that collaborators and I had worked on has been accepted for publication, which elucidates the responses of rat microglia to CNTF and to another member of the CNTF family, interleukin-6 (IL-6). Two additional manuscripts on the effects of IL-6 and CNTF on murine microglia have been prepared. One has been submitted to the Journal of Neurochemistry and the other will shortly be submitted to the Journal of Neuroinflammation. The PDF of the published manuscript has been provided as a supplemental file.

Results of experiments from Aim 1:

My studies showed that microglia possess functional CNTF receptor alpha (CNTFRa) and that microglia respond to CNTF stimulation by altering the intracellular phosphorylation of hematopoietic cell specific Lyn substrate-1 and beta-tubulin 5. Intriguingly, as interferon-gamma (IFNγ) is a major activator of the macrophage population, we found that IFNγ increases CNTFRa expression in microglia, which suggests that CNTFRa signaling may be involved in microglial responses to IFNγ.

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Moreover, CNTF stimulation induced mRNA and protein secretion of glial cell line derived neurotrophic factor (GDNF) in rat microgila.

Results of experiments from Aim 2:

My studies revealed that CNTF has immunological effects on microglia and that the soluble CNTFRa, which is a naturally occurring receptor for CNTF, plays an important role in CNTF function. The combination of CNTF and soluble CNTFRa, but neither alone, induced Cyclooxygenase-2 (Cox-2). Similarly, granulocyte macrophage colony stimulating factor (GMCSF)-induced dendritic-like cells derived from the microglia, only when CNTF was co-applied with soluble CNTFRa. Neither CNTF nor the soluble receptor alone induced CD40 expression. These results shed lights on how CNTF may be involved in the neuroinflammatory responses of the CNS and suggest a novel role for the soluble CNTFRa in regulating the immune system.

Results of experiments from Aim 3:

Unfortunately, we did not make satisfactory progress on completing aim 3. This was deliberate as the effects of CNTF on antigen presenting molecules was modest, and therefore, we predicted that we would not obtain robust results. Accordingly, rather than perform the experiments described in aim 3, we decided to determine how CNTF and IL-6 signal in microglia. Please refer to Project Challenges for results in the signaling studies.

Project Challenges

To our surprise, given that CNTF is known to utilize gp130 as s signal transducing receptor, we found that CNTF signals differently than IL-6 in both murine and rat microglia. In fact, we found that CNTF does not require gp130 to induce Cox-2 protein expression, which agreed with the absence of STAT and ERK activation after CNTF stimulation. Additional signaling pathways, such as AKT and p38 MAPK, were examined and found inactive upon treatment with CNTF. However, as we previously described that hematopoietic cell specific Lyn substrate-1 and beta-tubulin 5 are

1. **Hsiao-Wen Lin** and Steven W. Levison (2008). Context dependent IL-6 potentiation of interferongamma induced IL-12 secretion and CD40 expression in murine microglia (in preparation)
2. **Hsiao-Wen Lin**, Hong Li and Steven W. Levision (2008). Ciliary neurotrophic factor (CNTF) in combination with soluble CNTF receptor alpha increases cyclooxygenase-2 and potentiates IFNγ-induced CD40 expression in murine microglia (in preparation)

Reference

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Ciliary Neurotrophic Factor and Interleukin-6 Differentially Activate Microglia

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Studies have shown that cytokines released following CNS injury can affect the supportive or cytotoxic functions of microglia. Interleukin-6 (IL-6)-family cytokines are among the injury factors released. To understand how microglia respond to IL-6 family cytokines, we examined the effects of ciliary neurotrophic factor (CNTF) and IL-6 on primary cultures of rat microglia. To assess the functional state of the cells, we assayed the expression of tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), and cyclooxygenase 2 (COX-2) following stimulation. We show that CNTF reduces COX-2 levels, whereas IL-6 increases the expression of IL-1β, TNFα, and Cox-2. We also examined trophic factor expression and found that CNTF enhances glial cell-line derived neurotrophic factor (GDNF) mRNA and protein secretion, whereas IL-6 has no effect. Correspondingly, conditioned media from CNTF-stimulated microglia promote motor neuron survival threefold beyond controls, whereas IL-6-stimulated microglia decrease neuronal survival twofold. To understand better the signaling mechanisms responsible for the opposite responses of these IL-6-family cytokines, we examined STAT-3 and ERK phosphorylation in CNTF- and IL-6-stimulated microglia. IL-6 markedly increases STAT-3 and ERK phosphorylation after 20 min of treatment, whereas these signal transducers are weakly stimulated by CNTF across a range of doses. We conclude that CNTF modifies microglial activation to support neuronal survival and that IL-6 enhances their capacity to do harm, as a result of different modes of intracellular signaling. © 2008 Wiley-Liss, Inc.

Key words: IL-6; GDNF; inflammation; TNFα; COX-2; neurodegeneration

Microglia are widely regarded as cells with dual personalities. They can either function as support cells to promote neural cell survival and repair or function as phagocytes to destroy and remove cells undergoing degenerative changes. However, the molecular signals that determine which personality they will adopt remain only partially understood. Microglia are ubiquitous throughout the CNS and as such are well placed to support neurons and glia. Microglia can support cell survival by releasing growth factors such as fibroblast growth factor-2 (FGF-2), transforming growth factor-β1 (TGFβ1), and nerve growth factor (NGF; Bandtlow et al., 1990; Araujo and Cotman, 1992; Lu et al., 2005). Their broad distribution also enables them to eliminate cells efficiently through release of cytotoxic molecules, including nitric oxide (NO), reactive oxygen species (ROS), proinflammatory cytokines such as tumor necrosis factor-α (TNFα), and a number of proteolytic enzymes.

Although the responses of microglia in neurological diseases and injuries have been extensively studied, the mechanisms by which local cytokines alter their responses remain to be elucidated. In response to neuronal injury, microglia rapidly progress from a resting ramified state to an activated state in which they can proliferate, migrate, and increase or express de novo a number of growth and trophic molecules as well as

The first three authors contributed equally to this work.

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immune modulatory molecules (Frei et al., 1987; Lowe et al., 1989; Streit et al., 1989; Lenschow et al., 1996). If, however, neuronal injury is beyond repair and accompanied by cell death, microglia further differentiate into phagocytes to remove debris (Streit and Kreutzberg, 1988). The ability of these cells to undergo such drastic functional changes makes them critical in numerous neurological diseases, including multiple sclerosis (MS) and Alzheimer's disease (AD). Both of these neurodegenerative diseases have an inflammatory component that can lead to cellular degeneration, which may be exacerbated by a chronic or abnormal activation of microglia (Gonzalez-Scarano and Baltuch, 1999). As emphasized in a recent review, understanding how to shift microglia away from cell destruction and toward cell preservation has important ramifications for both neuroprotective and neuroregenerative therapies (Schwartz et al., 2006).

Interleukin-6 (IL-6)-family cytokines are induced in response to brain injury. Members of this cytokine family include IL-6, leukemia inhibitory factor (LIF), cardiotrophin-1, oncostatin M, IL-11, and ciliary neurotrophic factor (CNTF). Astrocytes, microglia, and neurons are sources of IL-6 in the brain, and IL-6 is a potent microglial activator stimulating the production of COX-2, NO, ROS, and proteolytic enzymes. IL-6 also causes abundant expression of TNFα, which has been directly implicated in demyelination (Selmaj and Raine, 1988; Selmaj et al., 1991).

The damaging effects of IL-6 contrast with those of its related family member, CNTF. Whereas IL-6 elicits proinflammatory responses, CNTF is widely viewed as a regenerative cytokine acting both directly and indirectly. CNTF is produced following brain injury by astrocytes and directly supports the survival of a variety of neuronal populations, including motor neurons both in vitro and in vivo (Arakawa et al., 1990; Sendtner et al., 1990; Sendtner et al., 1992; Hagg and Varon, 1993; Dale et al., 1995; Lo et al., 1995; MacLennan et al., 1996). In addition, CNTF activates astrocytes to enhance their capacity to support neuronal and oligodendroglial survival (Albrecht et al., 2002, 2007). However, the effects of CNTF on microglia have been only partially studied (Hagg et al., 1993; Kahn et al., 1995; Martin et al., 2003). We show here that, consistent with the view that CNTF promotes cell survival, in addition to its direct effects on neurons, CNTF shifts microglia to enhance their capacity to support neuronal survival.

MATERIALS AND METHODS

Materials

Rat recombinant CNTF (rrCNTF) was purchased from Alomone (Jerusalem, Israel; C-245) and rat recombinant IL-6 (rrIL-6) was purchased from R&D Systems (Minneapolis, MN; 506-RL-10). Antibodies for COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CNTFRα antibody was purchased from R&D Systems. Anti-STAT-3, phospho-STAT-3(tyr705), phospho-ERK 1/2, and ERK 1/2 primary antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA). Rabbit polyclonal antibody against choline acetyltransferase (ChAT) was purchased from Chemicon (Temecula, CA).

Primary Glial Cultures

Primary mixed glial cultures were prepared from postnatal day 2 rat cerebral cortex as described previously (Levison and McCarthy, 1991). Briefly, Sprague-Dawley rat pups were sacrificed by decapitation, and the cerebral cortices were isolated. The meninges were removed, the tissue was enzymatically and mechanically dissociated, and the cell suspension was passed through nylon mesh screens. Cells were counted using a hemocytometer in the presence of 0.1% trypan blue and plated into 75-cm^2 tissue culture flasks at a density of 1.5×10^7 viable cells per 75-cm^2 flask in minimum essential medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/100 μg/ml penicillin/streptomycin, and 0.6% glucose (MEM-C). Medium was changed every 2 days after plating. At confluence, the cultures were shaken on an orbital shaker at 260 rpm for 90 min to dislodge microglial cells. The nonadherent cells were plated onto plastic petri dishes, and the dishes were placed in a 37°C incubator for 40 min to allow the microglial cells to adhere. The dishes were rinsed extensively with MEM to eliminate nonadherent cells. Cells were detached using Accutase (Innovative Cell Tech) and replated onto six-well plates (10^5 cells/cm^2) in a defined medium containing MEM supplemented with 0.66 mg/ml BSA, 100 μg/ml d-biotin, 5 ng/ml insulin, 1 ng/ml selenium, 40 μg/ml iron-poor transferrin, 2 mM glutamine, 15 mM HEPES buffer, 100 U/100 μg/ml penicillin/streptomycin, and 1% FBS (MCDM). Before treatment with cytokines, these microglia-enriched cultures were maintained for 1 day. Microglial culture purity was determined by CD11b and A2B5 staining as 90% microglia.

Motor Neuron Isolation

Spinal cords were collected from embryonic day 15 (e15) rat fetuses. Metrizamide centrifugation was used to isolate specifically a band of large cells enriched in motor neurons (Henderson et al., 1994). Viable cells were determined by trypan blue exclusion and the cells were seeded at 5×10^3 cells/cm^2 in defined medium (MN1A) containing 2% horse serum on laminin/poly-D-lysine-coated coverglasses (Albrecht et al., 2002). After a 24-hr incubation allowing the motor neurons to attach, the medium was aspirated and fresh medium, microglial conditioned medium (MCM), CNTF MCM, or IL-6 MCM was added to each condition and incubated for 2 days. After 2 days, cells were fixed with 3% paraformaldehyde and incubated with rabbit polyclonal antibody against ChAT (1/500 dilution) at 37°C for 1 hr. Cells were rinsed and incubated with appropriate fluorochrome-conjugated secondary antibodies at 37°C for 1 hr, followed by DAPI counterstain. Cells were then rinsed in PBS, mounted onto glass coverslips and counted by two investigators.

RNA Isolation

Medium from microglia in tissue culture plates was aspirated, and cells were placed in a −80°C freezer until RNA isolation. RNA was isolated from cells grown in monolayer using Trizol from Molecular Research Center, Inc. (Cincinnati, OH), according to the manufacturer's protocol. RNA was resuspended in RNase-free water, and the absorbance was taken using a Beckman D464 spectrophotometer.

Protein Isolation

Microglial cells were washed twice with ice-cold PBS, then lysed in buffer containing a final concentration of 1% Triton X-100, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 0.2% EGTA, 0.2% sodium orthovanadate, and protease inhibitor cocktail (Sigma, St. Louis, MO). DNA was sheared using a 21-gauge needle, and the lysate was incubated on ice for 30 min prior to centrifugation at 10,000*g* for 15 min at 4°C. Protein levels from these supernatants were determined using the BCA colorimetric assay (Pierce, Rockford, IL). Protein lysates were aliquoted and stored at −20°C until needed.

Enzyme-Linked Immunosorbent Assay

GDNF content in the supernatant was assayed by enzyme-linked immunosorbent assay (ELISA) developed with commercially available matching pairs of antibodies and appropriate standards purchased from R&D Systems (antibody pairs, BAF212 and MAB212, and recombinant GDNF, 212-GD). Microglia were plated at a density of 1.5×10^5 cells/cm^2 on 60-mm dishes with 2 ml MCDM. Cells were stimulated with rrCNTF (5 ng/ml) or left untreated for 24 hr. Supernatants were collected and concentrated using Centricon centrifugal filter devices purchased from Millipore (Bedford, MA; YM-10). TNFα was assayed by ELISA developed with the commercially available matching pairs of antibodies and appropriate standards purchased from R&D Systems.

Real-Time RT-PCR

Two micrograms of RNA from each condition was reverse transcribed to cDNA using the Omniscript RT Kit from Invitrogen. Real-time PCR reactions were performed with 50 μl platinum qPCR Supermix UDG Kit from Invitrogen (Carlsbad, CA). Taqman primers were purchased from ABI (Foster City, CA) for murine IL-1β, TNFα, and 18S RNA (catalog Nos. Rn00580432, Rn99999017, and Hs99999901, respectively). The LUX Designer Program from Invitrogen was used to generate a forward and reverse primer pair for rat GDNF (accession No. NM109139): CACAGG TCGATATTGTAGCGGTTCCTGTG (forward), TGCCTG GCCTACCTTGTCACT (reverse). An unlabeled forward 18S mRNA primer and JOE-labeled reverse 18S mRNA primer were purchased from Invitrogen, both of which are compatible with human, mouse, or rat 18S rRNA. Reactions for LUX primers included 2 μl template cDNA, 12.5 μl Supermix, 0.5 μl forward primer, 0.5 μl reverse primer, 0.5 μl ROX, and 9 μl RNase-free water. An Applied Biosystems ABI Prism 7700 SDS PCR instrument was employed for analyses. The cycling conditions were as follows: hot start (95°C for 2 min), PCR cycling (95°C for 15 sec for denaturation, 55°C for 30 sec for annealing, 72°C for 30 sec for extension, with a fluorescence measurement at the end of the extension step), repeated 45 times. Data were analyzed using SDS 1.9 software from Applied Biosystems and the Relative Expression Software Tool (REST) for groupwise comparison and statistical analysis of relative expression results in real-time PCR (Pfaffl, 2001).

Western Blot Analysis

Fifteen micrograms of protein isolated from the microglial preparations was separated on 8% polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes. Membranes were dried, stained with 0.2% Ponceau S for 10 min (Sigma), and then destained in 5% acetic acid. Images of total protein were then captured, whereupon the membranes were blocked for 1 hr in blocking solution (Roche, Indianapolis, IN), rinsed briefly in PBS, and incubated overnight at 4°C in primary antibody diluted 1/10 in blocking solution or 1% BSA diluent. After incubation with the primary antibody, the blot was extensively washed in PBS-Tween and then incubated for 2 hr at room temperature with secondary antibody conjugated to HRP. The membrane was then washed extensively in PBS-Tween prior to visualization using Rennaisance Chemiluminescence (NEN Life Science Products, Boston, MA). For COX-2 analysis, anti-COX-2 primary antibody was diluted 1/1000. For CNTFRα analysis, anti-CNTFRα antibody was diluted 1/500, and HRP-conjugated donkey anti-goat antibody was used at 1/20,000 dilution. For STAT-3 Western analysis, anti-STAT-3 primary antibody was diluted 1/1,000, and HRP-conjugated donkey anti-rabbit secondary antibody was used at 1/10,000. Images were obtained and quantified using a UVP imaging system with LabWorks software (UVP, Upland, CA).

Statistical Analysis

Comparisons of protein data and cell survival assays were performed using a two tailed Student's *t*-test to detect *P* values of 0.05 between the means of the treatment groups and untreated controls. For real-time PCR data, the REST program was used to detect significant difference between the means of treatment group and untreated control based on *P* values of 0.05 (Pfaffl, 2001).

RESULTS

CNTFRα Expression in Primary Cultured Rat Microglia

To determine whether microglia have the capacity to respond directly to CNTF, we evaluated CNTFRα expression by Western blot. Protein lysates were generated from microglia isolated from newborn mixed glial cultures, type 1 astrocytes, and meningeal fibroblast cultures. Astrocytes and fibroblast homogenates were included because both cell types are known to express low levels of CNTFRα (Monville et al., 2001). Spinal cord homogenates as well as lysates from the human neuroblastoma cell line SH-SY5Y were included as positive references because these samples are known to con-

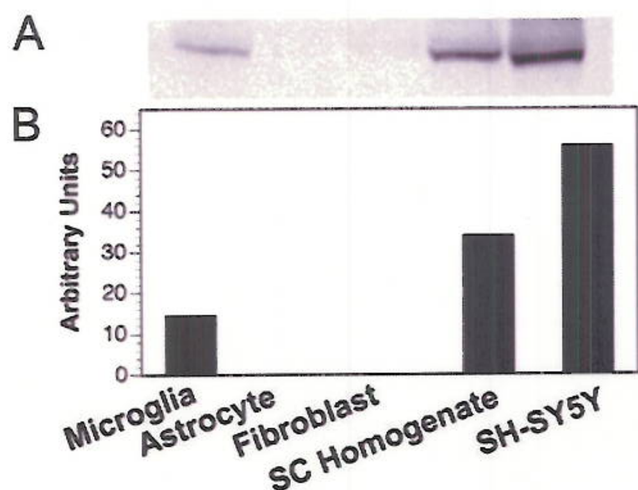

Fig. 1. CNTFRα is expressed by cultured rat microglia. **A:** Western blot using an anti-CNTFRα antibody on 10 μg total cell homogenates from rat primary microglia (lane 1), rat astrocytes (lane 2), rat fibroblasts (lane 3), rat spinal cord homogenate (lane 4), and SH-SY5Y cells (lane 5). **B:** Densitometric analysis of the above Western blot shows CNTFRα expression (in arbitrary units) using a UVP chemi-imaging system.

tain high levels of CNTFRα (Davis et al., 1991). This analysis revealed that rat microglia express the α subunit of the receptor for CNTF (Fig. 1). Moreover, cultured microglia expressed the CNTFRα whereas it was undetectable on astrocytes and fibroblasts. Levels of the CNTFRα in microglia were lower than those in spinal cord and neuroblastoma homogenates. Microglia are known to express gp130 and the LIF receptor β, so these results suggest that microglia are capable of directly responding to CNTF.

Cytokines Differentially Affect Microglial Expression of Cyclooxygenase-2

Workers in our laboratory have previously used expression of the enzyme COX-2 as an index of microglial activation (Basu et al., 2002b, 2005). To date, the effects of CNTF on COX-2 expression in microglia have not been evaluated. Primary rat microglia were stimulated with 10 ng/ml rrIL-6 or 1 ng/ml rrCNTF (concentrations chosen to approximate their ED_{50} based on previous studies) for 16 hr, and then COX-2 protein levels were analyzed by Western blot (Fig. 2). Untreated microglia had a basal level of COX-2 protein. Upon stimulation with IL-6, COX-2 protein levels increased 6.4-fold compared with untreated levels. By contrast, CNTF decreased microglial COX-2 levels 1.5-fold compared with the untreated cultures. Changes in COX-2 mRNA levels at 6 hr of treatment mirrored these changes in protein levels, suggesting that these cytokines affect COX-2 mRNA transcription (data not shown).

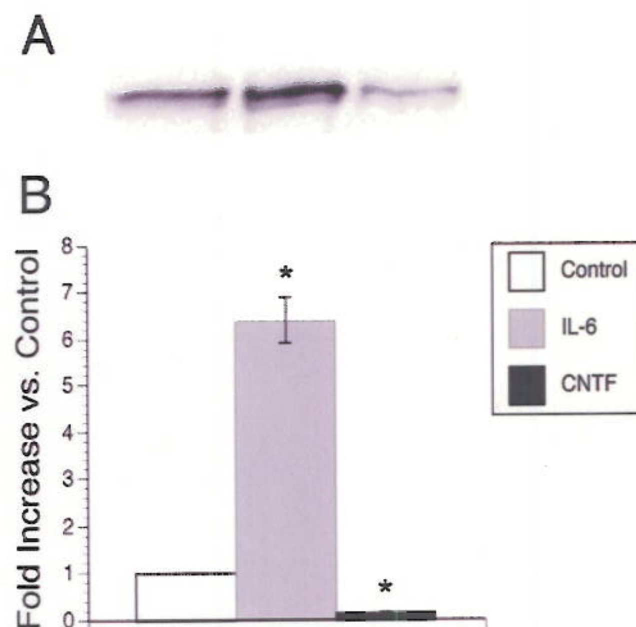

Fig. 2. Microglial COX-2 protein levels are differentially altered in response to IL-6-family cytokines. **A:** Primary rat microglia were stimulated with rrIL-6 (10 ng/ml) or rrCNTF (1 ng/ml) for 16 hr. After cytokine treatment, 20-μg protein lysates were examined by Western blot analysis with a COX-2 primary antibody. Equivalent protein loading was confirmed by Ponceau S staining (data not shown). **B:** Western analysis data are represented as -fold increase over control. Quantification was performed using a UVP chemi-imaging system. Data are averaged from three separate experiments. Values are means ± SEM. $^{*}P < 0.05$ by Student's *t*-test vs. control.

IL-6 but Not CNTF Stimulation Increases Microglial mRNA Expression of Proinflammatory Cytokines

To characterize further the effect of these cytokines on microglial activation, we assayed levels of microglial mRNA for proinflammatory cytokines by real-time PCR. Microglial cultures were stimulated with either rrIL-6 or rrCNTF for 8 hr. After stimulation, total RNA was isolated, reverse transcribed, and used as template in real-time PCR assays. Primers to the proinflammatory cytokines TNFα and IL-1β were used to assay mRNA levels for these cytokines following stimulation, and primer pairs to 18s RNA were used for normalization. Figure 3A is a graphic representation of the results of the real-time PCR experiments. IL-6 increased levels of mRNAs for both TNFα and IL-1β compared with control cultures. TNFα mRNA expression increased 8-fold over untreated controls, whereas IL-1β mRNA expression increased 14-fold. CNTF stimulation, by contrast, was without effect on either TNFα or IL-1β mRNA expression.

To assess whether IL-6 also increased cytokine release, primary rat microglia were treated with CNTF and IL-6 as described above. Supernatants were isolated from the stimulated cultures and analyzed for TNFα

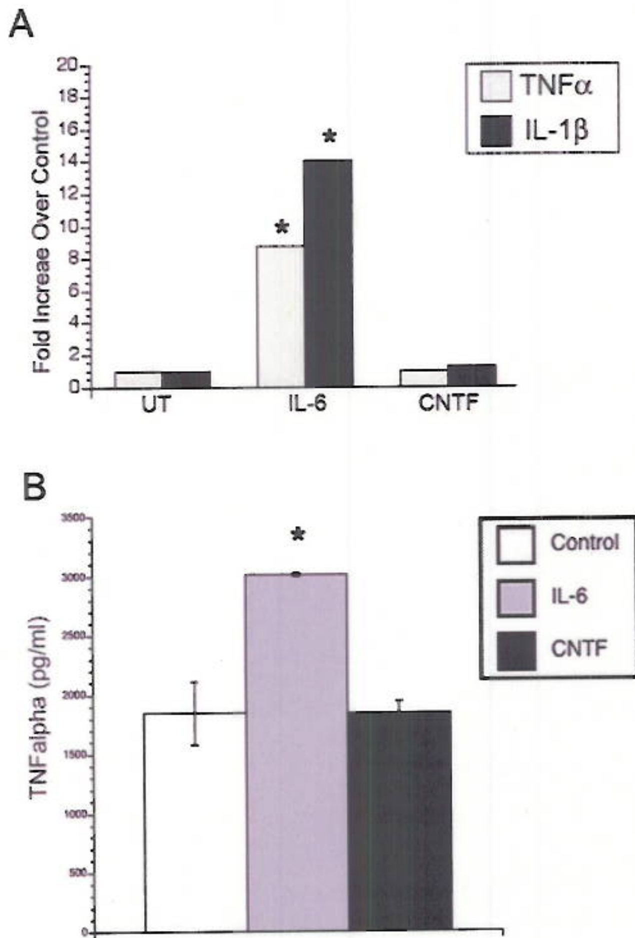

Fig. 3. IL-6, but not CNTF, stimulates microglial proinflammatory cytokines. **A:** Microglia were untreated (UT) or were stimulated with rrIL-6 (10 ng/ml) or rrCNTF (1 ng/ml) for 8 hr. Total RNA was isolated, reverse transcribed, and used for real-time PCR analyses for TNFα and IL-1β with a primer to 18s RNA for normalization. IL-6-stimulated mRNAs for both TNFα ($P < 0.05$) and IL-1β ($P < 0.01$) were compared with control cultures, whereas CNTF was without effect for either TNFα or IL-1β. Data are representative of three independent experiments. Statistical significance was determined using the REST program. **B:** Microglia were treated with rrCNTF (5 ng/ml) or rrIL-6 (5 ng/ml) or were untreated for 24 hr. Supernatants were collected and analyzed by ELISA for TNFα secretion. Data are averaged from three independent experiments. Values are means ± SEM. $^{\star}P < 0.01$ by Student's *t*-test vs. control.

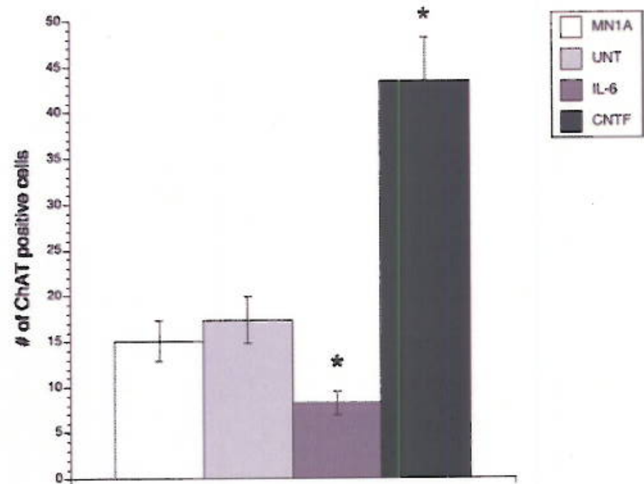

Fig. 4. CNTF-stimulated microglia promote motor neuron survival. Microglia were pretreated for 6 hr with MN1A, rrIL-6 (10 ng/ml), or rrCNTF (1 ng/ml) or were untreated. Cultures were then rinsed extensively and incubated with MN1A for 2 days to condition the media. The conditioned media were then introduced to motor neuron cultures for 2 days, after which the motor neurons were immunohistochemically stained with antibodies against choline acetyltransferase (ChAT) to identify motor neurons positively. Each condition was assayed in triplicate, with cell counts from three fields per sample averaged. Cell counts were performed by two independent investigators. Data are averaged from three independent experiments. Values are means ± SEM. $^{\star}P < 0.05$ vs. control by Student's *t*-test vs. control.

secretion by ELISA. After IL-6 treatment, TNFα levels in the medium were twice the level seen in control cultures (Fig. 3B). By contrast, CNTF did not affect the release of TNFα from microglia.

CNTF-Treated Microglia Promote Motor Neuron Survival

Depending on their functional state, microglia can either promote neuronal survival or stimulate neuronal demise. To assess how these IL-6-family cytokines affect the functional state of microglia, we examined the effects of cytokine-stimulated microglia on motor neuron survival. Microglia were stimulated with CNTF or IL-6 for 6 hr, after which the microglia were rinsed three times with MEM and then incubated with hormone-supplemented media for 2 days to allow factors produced by the microglia to accumulate in the media. This "conditioned" media was then introduced to primary motor neuron cultures for 2 days, after which the motor neurons were fixed and stained with antibodies against ChAT to determine the number of surviving neurons. CNTF-stimulated microglial conditioned media (MCM) enhanced the survival of spinal cord motor neurons approximately threefold compared with motor neurons exposed to media conditioned from unstimulated microglia (Fig. 4). By contrast, motor neuron survival was reduced twofold with maintenence in IL-6 MCM. Because the microglial cultures were extensively rinsed prior to conditioning, a direct effect of the cytokines does not account for the changes in motor neuron survival. Moreover, we previously established that CNTF is not a potent motor neuron survival factor (Albrecht et al., 2002).

CNTF Increases GDNF mRNA Expression and Protein Secretion in Microglia

The increased motor neuron survival in media conditioned from CNTF-stimulated microglia suggested that CNTF elicited the secretion of neurotrophic factors

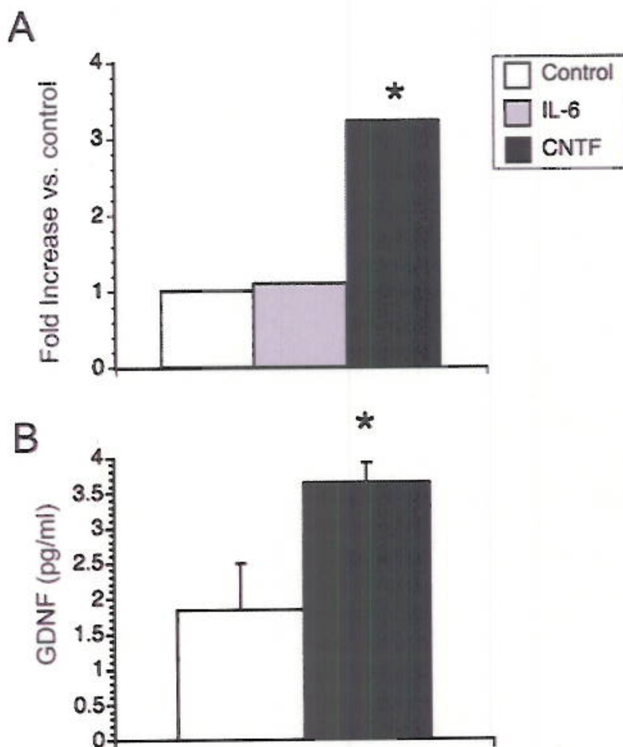

Fig. 5. GDNF expression is increased following CNTF but not IL-6 treatment. **A:** Microglia were stimulated with rrCNTF (5 ng/ml) or rrIL-6 (5 ng/ml) or were untreated for 6 hr. After washing of the cells, total RNA was isolated and used to generate templates for real-time PCR. Primers to 18s were used to correct RNA amounts within individual samples. Samples were assessed in duplicate, and results were averaged from three independent experiments. $^{*}P < 0.05$ using the REST program. **B:** Microglia were stimulated with rrCNTF (5 ng/ml) or left untreated for 24 hr. Supernatants were collected, concentrated, and analyzed by ELISA for GDNF secretion. The data contain four samples in each condition and are representative of three independent experiments. Values are means ± SEM. $^{*}P < 0.05$ by Student's *t*-test vs. control.

from microglia. One possible candidate, GDNF, is a potent neurotrophic factor for motor neurons. To determine whether microglia produce GDNF in response to CNTF, we analyzed mRNA expression from microglial cultures exposed to either IL-6 or CNTF by real-time PCR. Primary rat microglia were stimulated with either CNTF or IL-6 for 6 hr. After cytokine treatment, total RNA was isolated and used to generate templates for real-time PCR. As depicted in Figure 5A, CNTF increased GDNF mRNA 3.2-fold compared with untreated microglia. By contrast, IL-6 had no effect on microglial GDNF mRNA expression, with a trend across experiments toward decreasing GDNF mRNA expression. We also assessed GDNF protein secretion. Because the production of GDNF is typically low, concentrating the supernatants prior to ELISA analysis was required. CNTF increased GDNF levels in the medium in accordance with the increase in mRNA levels (Fig. 5B). The levels of BDNF, another motor neuron trophic factor, did not increase significantly at the mRNA level after 6 hr treatment with these cytokines (data not shown).

STAT-3 Is Weakly Phosphorylated Following CNTF Treatment

To understand better how CNTF and IL-6 elicit different responses from microglia, we examined the phosphorylation status of STAT-3, a transcription factor that is involved in the signal transduction cascade activated by both cytokines in various cell types. Microglial cultures were treated with either IL-6 or CNTF for 0, 5, 20, and 40 min, and the levels of the band that migrated at ~90 kDa when stained using an antibody to STAT-3 or a phospho-STAT-3 antibody were analyzed by Western blot. IL-6 elicited a pronounced increase in STAT-3 phosphorylation by 20 min, with a slight drop off from peak value at 40 min (Fig. 6A). By contrast, CNTF weakly elicited STAT-3 phosphorylation at 5 or 20 min, with no effect evident at 40 min. In some experiments, CNTF had no detectable effect on STAT-3 phosphorylation. We also examined phosphorylation of ERK, because it is also a common signaling pathway downstream of IL-6-family cytokines. The pattern of ERK phosphorylation was similar for CNTF and IL-6 (Fig. 6B). The weaker phosphorylation of STAT-3 by CNTF was not because of the low dose of CNTF used, because up to 50 ng/ml of CNTF still failed to increase phosphorylation of STAT-3 as strongly as IL-6 (Fig. 6C). Dose-effect experiments confirmed the observation that CNTF weakly stimulated ERK phosphorylation (Fig. 6D). Interestingly, CNTF stimulated only ERK-2 and not ERK-1. With STAT-3 phosphorylation as an index for CNTF stimulation, these dose-effect experiments demonstrated that CNTF achieved 50% maximal effect at 0.3 ng/ml. Similarly, with STAT-3 phosphorylation as an index for IL-6 stimulation, these dose-effect experiments demonstrated that 3 ng/ml IL-6 achieved half-maximal stimulation, and the peak of stimulation was achieved at 50 ng/ml. Thus, for most of the experiments in the studies reported here, each cytokine was used at approximately three times its ED_{50}.

DISCUSSION

Microglia may positively or negatively affect neuronal survival. Here we demonstrate that two members of the IL-6 family of cytokines have distinct and opposing effects on the functional properties of microglia that in turn have important consequences for motor neuron survival. CNTF reduces COX-2 mRNA and protein levels, enhances GDNF mRNA and protein secretion, and has no effect on TNFα or IL-1β production. Consistent with CNTF altering the state of microglia, motor neuron survival is increased when the neurons are maintained in medium conditioned by CNTF-stimulated microglia. The effects observed with IL-6 were all opposite those obtained following CNTF treatment. IL-6 increases levels of COX-2 and increases TNFα and IL-

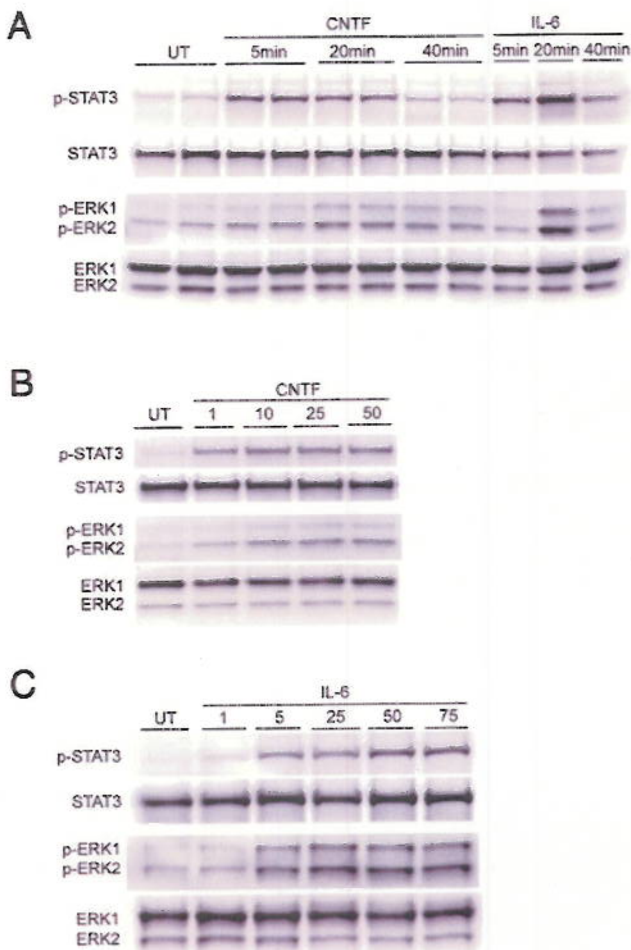

Fig. 6. IL-6 induces strong phosphorylation of STAT-3, whereas CNTF weakly stimulates STAT-3 in microglia. Microglia were treated with rrIL-6 or rrCNTF or left untreated (UT). After treatment, 15 μg protein lysate from each sample was analyzed by Western blot. **A** shows representative Western blots for phosphorylated STAT-3, total STAT3, phosphorylated ERK1/2, and total ERK1/2. Microglia were treated with CNTF (10 ng/ml) or IL-6 (5 ng/ml) for 0, 5, 20, and 40 min. **B** shows representative Western blots for phosphorylated STAT-3, total STAT3, phosphorylated ERK1/2, and total ERK1/2 at 20 min after CNTF 1, 10, 25, and 50 ng/ml stimulation. **C** shows representative Western blots for phosphorylated STAT-3, total STAT3, phosphorylation ERK1/2, and total ERK1/2 at 20 min after IL-6 1, 5, 25, 50, and 75 ng/ml stimulation. Data are representative of three independent experiments.

1β production, and conditioned medium from IL-6-treated microglia is neurotoxic. We also find unexpected differences within the cardinal signaling pathway traditionally associated with these receptors; notably, STAT-3 and ERK are weakly phosphorylated following CNTF treatment, whereas they are rapidly and highly phosphorylated after IL-6 stimulation.

Several studies implicate IL-6 as a strong microglial activator. For instance, studies on cultured rat microglia have shown that IL-6 stimulates microglial proliferation (Streit et al., 2000). Other studies have evaluated markers for microglial activation and found them to be induced by IL-6. Markers used to date include granulocyte-macrophage colony-stimulating factor (GM-CSF), metallothionines, COX-2, and the protein tyrosine kinases Hck, Fes, and Fak (Basu et al., 2002a; Krady et al., 2002). Similarly, transgenic mice overexpressing IL-6 in the brain develop severe neurologic symptoms characterized by tremor, ataxia, and seizures. These IL-6-overexpressing transgenic mice also show deficits in avoidance learning that parallel the degenerative changes seen histologically (Heyser et al., 1997). Furthermore, increased levels of several acute-phase proteins, such as α1-antichymotrypsinogen, are observed (Campbell et al., 1993). Complementary studies in IL-6 null mice report that microglial activation is severely abrogated after cortical and facial nuclei lesions (Klein et al., 1997; Penkowa et al., 1999). Furthermore, IL-6 can induce the expression of other neurotoxic mediators. TNFα has been implicated in a number of CNS diseases, including MS and AD (Hofman et al., 1989). TNFα is toxic to motor neurons (Ghezzi and Mennini, 2001), is also toxic to oligodendrocytes, and has been implicated in demyelination (Powell et al., 1990; Benveniste, 1997). Here we examined TNFα production by microglia following CNTF and IL-6 treatment and found that microglia treated with CNTF failed to induce TNFα protein, whereas IL-6 stimulated a twofold increase in TNFα protein secretion.

Supporting data for the hypothesis that IL-6 activates microglia but CNTF shifts their activation come from the present analysis of COX-2 production. COX-2 is a highly inducible enzyme that catalyzes the conversion of arachidonic acid to inflammatory mediators, including prostanoids, which can contribute to edema and inflammation (Minghetti et al., 1999; Koistinaho and Chan, 2000; Smith et al., 2000). Reactive microglia also produce reactive oxygen species that promote oxidative stress, which, when combined with edema and inflammation, provide an environment that will exacerbate tissue damage and/or cell death. These byproducts are known toxins to motor neurons. The accumulation of COX-2-derived inflammatory mediators accompanies a number of neurodegenerative diseases, including MS, experimental allergic encephalomyelitis (EAE; the animal model of MS), amyotrophic lateral sclerosis (ALS), and AD (Lipsky, 1999; Aloisi et al., 2000; Almer et al., 2001; Yasojima et al., 2001; Rose et al., 2004). Prostaglandin E_2 (PGE_2), a COX-2 derivative, exacerbates motor neuron loss and is implicated in ALS pathogenesis (Almer et al., 2001; Yasojima et al., 2001), and COX-2 inhibitors prevent motor neuron death in vitro (Klivenyi et al., 2004).

It is important to keep in mind that IL-6 and CNTF are expressed and released at different time points following brain damage. In general, IL-6 is produced early following brain injury and prior to microglial activation. For instance, in a rat seizure model, IL-6 is released 10 hr postseizure, prior to maximal microglial activation at 24 hr (Rosell et al., 2003). Work from our

laboratory has shown that the proinflammatory cytokine IL-1β (which we have shown is upstream of IL-6) is expressed in the spinal cord during the demyelination stage after MHV-A59 infection (by 1 week of infection), whereas CNTF is elevated during the remyelination phase at 4 weeks postinfection (Albrecht et al., 2003). These data support the hypothesis that IL-6 is responsible for cell damage following CNS injury resulting from microglial activation, whereas the delayed expression of CNTF promotes recovery by shifting microglial toward a more nurturing state. Indeed, the courses of demyelinating diseases are exacerbated by null mutations in the CNTF gene. In a study of 288 patients with MS, Giess et al. (2002) found that the patients with CNTF null mutation developed symptoms at 17 years of age vs. 28 years in CNTF heterozygotes and that the patients who have no CNTF production had more severe motor disabilities and more relapses than control patients. In a complementary study performed on mice with a genetic deletion of CNTF, Linker et al. (2002) found that the course of MOG-induced EAE was more severe and that recovery was slower in the CNTF null mice than in wild-type controls. Mice without CNTF developed motor symptoms 3 days earlier, and all of the CNTF null mice suffered from relapses, whereas only 36% of the wild-type mice had relapses. Histological analyses revealed that there was increased myelin splitting and vacuolar myelin degeneration in the null mice as well as increased axonal pathology. An analysis of apoptosis demonstrated that there was significantly more apoptosis in the spinal cord throughout the disease course in the CNTF nulls. The authors concluded that CNTF acts on oligodendrocytes and neurons directly to protect them from sustaining damage and that, in its absence, these cells are more vulnerable. Our data suggest that the absence of CNTF would alter microglial reactivity (and perhaps macrophage reactivity), resulting in an exacerbation of the disease course.

CNTF can directly rescue chick embryonic spinal motor neurons, but CNTF-stimulated astrocyte conditioned medium also rescues motor neurons, indicating that CNTF can act indirectly (Yin et al., 1994). As we showed previously, CNTF increases the production and secretion of FGF-2 from astrocytes, which is a known motor neuron survival factor (Albrecht et al., 2002). Here we show that CNTF also promotes survival by recruiting microglia to increase their production of trophic factors. We observed a two- to threefold increase in microglial GDNF mRNA expression and protein production following CNTF treatment, whereas IL-6 failed to elicit GDNF expression. Although the production of GDNF by microglia has been reported previously, and stimulated release of GDNF after ischemia also has been shown, to date the stimulus responsible for inducing GDNF production by microglia has not been reported (Lee et al., 2004; Lu et al., 2005). At this time, it is premature to conclude that the production of GDNF by the microglia is wholly responsible for the survival effects of CNTF-treated microglia. In a microarray screen that we used to identify potential survival-promoting factors, we found that CNTF and IL-6 each altered the expression of over 80 genes, with approximately equal numbers of genes increased and decreased in expression. A subset of those genes that are relevant to the data reported here that were increased by CNTF but not IL-6, or visa versa, are listed in Supplemental Table I. These data support the conclusion that these cytokines shift many of the properties of microglial cells, rather than simply affecting one or two functions. That said, however, a role for GDNF is well supported by studies showing that GDNF is a potent motor neuron survival factor (Henderson et al., 1994; Schaar et al., 1994; Bohn, 2004) and by data showing that GDNF prevents motor neuron degeneration in a murine model of ALS (Wang et al., 2002).

How is it that CNTF and IL-6 have opposite effects on microglia when they are members of the same cytokine family? IL-6-family cytokines have been presumed to activate similar signaling cascades. After binding to their receptors leading to homo- or heterodimerization of gp130 subunits, they activate phosphorylation of downstream signaling transducers, including cytoplasmic STATs. Phosphorylated STAT monomers then dimerize and translocate to the nucleus to modulate gene transcription. We hypothesized that differential phosphorylation of STAT-3 could be responsible for the opposite effects of CNTF and IL-6 on microglial activation. Consistently with this hypothesis, IL-6 stimulated a rapid phosphorylation of STAT-3 as well as ERK and an activated microglial phenotype. By contrast, CNTF only weakly stimulated STAT-3 and ERK phosphorylation and shifted the microglial phenotype toward a growth-promoting state. Several signaling molecules have been implicated in modifying microglia. In particular, the antiinflammatory cytokines IL-4, IL-10, vasoactive intestinal peptide, and pituitary adenylate-activating polypeptide all increase levels of cyclic AMP to increase CREB phosphorylation, leading to less microglial activation (Delgado et al., 2002). To date, it is not known whether CNTF increases levels of cyclic AMP.

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