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# Peripheral nerve induces macrophage neurotrophic activities: regulation of neuronal process outgrowth, intracellular signaling and synaptic function

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**Abstract**

Rat cortical neurons cultured in conditioned media from human monocyte-derived macrophages (MDM) show increased neuronal protein synthesis, neurite outgrowth, mitogen-activating protein kinase activity, and synaptic function. Neurotrophic properties of human MDM-conditioned media are significantly enhanced by human peripheral nerve and to a more limited extent by CD40 ligand pre-stimulation. Such positive effects of MDM secretions on neuronal function parallel the secretion of brain-derived neurotrophic factor (BDNF). MDM activation cues may serve to balance toxic activities produced during neurodegenerative diseases and thus, under certain circumstances, mitigate neuronal degeneration.


*Keywords:* Monocyte-derived macrophages; Neurotrophins; Signal transduction

## 1. Introduction

Central nervous system (CNS) innate immune responses play a fundamental role in health and disease. Brain macrophages and T-cell lymphocytes mediate inflammatory responses that facilitate both neurotoxic and neurotrophic processes in the CNS. In the environment of CNS degenerative disease and traumatic injury, macrophages secrete inflammatory cytokines, alpha and beta chemokines, quinolinic acid, arachidonic acid and its metabolites, nitric oxide and free radicals (Gendelman, 2002; Kiefer et al., 2001) as well as growth inhibiting proteins such as chondroitin sulfate proteoglycans (Al-Omaishi et al., 1999; Cotter et al., 1999; Epstein and Gelbard, 1999; Fitch and Silver, 1997; Gendelman, 2002; Jansen and Reinhard, 1999; McGeer and McGeer, 1999). Secretion of inflammatory cytokines and growth-inhibiting proteins by activated immune cells has been shown to cause secondary degeneration following cell death caused by disease or trauma (Popovich et al., 2002; Popovich and Jones, 2003).

Specific environmental activation cues present during neurodegenerative disease and CNS injury may also be responsible for neurotrophic responses. A growing body of evidence suggests that CNS innate inflammatory responses promote neuronal process outgrowth and functional recovery following injury (Schwartz, 2002). As observed in the peripheral nervous system, macrophages, once recruited to the site of CNS injury, promote phagocytosis of myelin debris and recycling of lipid degradation products required for the repair and regeneration of damaged neurons and their processes (Lotan and Schwartz, 1994). In addition, macrophages secrete a wide range of cytokines and growth factors with neurotrophic properties

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including nerve growth factor (NGF) (Caroleo et al., 2001; Elkabes et al., 1996), brain-derived neurotrophic factor (BDNF) (Batchelor et al., 1999, 2002b; Dougherty et al., 2000), glial-derived neurotrophic factor (GDNF) (Batchelor et al., 1999, 2002b; Satake et al., 2000; Wei et al., 2000; Yamamoto et al., 1998), growth/differentiation factor-15/macrophage inhibitory cytokine (GDF-15/MIC) (Strelau et al., 2000) and interleukin-6 (IL-6) (Kiefer et al., 2001). Moon et al. (2002) recently showed that macrophages support the synthesis of neuronal growth promoting heparan sulfate proteoglycans while microglia and astrocytes synthesize growth inhibiting chondroitin sulfate proteoglycans. In toto, macrophage secretory and substrate bound proteins can increase neurite output and branching (Patrick et al., 1996), activate mitogen-activated protein kinase (MAPK) pathways and increase choline acetyltransferase (ChAT) activity (Jonakait et al., 2000). Upregulation of cellular processes such as these is necessary for neuronal differentiation or recovery from injury (Jonakait et al., 2000).

Further, in vivo studies have shown that enhanced sprouting of dopaminergic neurons following striatal injury directly corresponds to the recruitment of activated brain macrophages (Batchelor et al., 2002a). Implantation of peripheral macrophages into regions of damaged spinal cord results in degradation of growth inhibiting myelin proteins and production of regenerative extracellular matrix molecules (Franzen et al., 1998). Partial restoration of motor function has been shown to occur when macrophages and T-cell lymphocytes are activated with peripheral nerve (PN) and subsequently injected into areas of spinal cord injury (Prewitt et al., 1997; Rapalino et al., 1998; Schwartz, 2000; Schwartz et al., 1999a; Schwartz and Moalem, 2001; Zeev-Brann et al., 1998). Recent work has begun to characterize the role of T cell lymphocytes in the neurotrophic immune response. T cell mediated protective responses induced by active or passive immunization of CNS-injured rats with myelin-associated peptides or Nogo A (Hauben et al., 2000, 2001) protect injured axons from secondary degeneration by inducing neurotrophin production by surrounding astrocytes and macrophages (Barouch and Schwartz, 2002). Clearly, T lymphocytes play a major role activating neuroprotective and neuroregenerative processes in the damaged CNS, however, the role of macrophages has not been defined.

Given that environmental cues can influence the balance of neurotoxic and neurotrophic properties of macrophages, we developed in vitro cellular assays to assay the pathways for neuroregeneration. In this regard, we hypothesized that CD40 ligand (CD40L) and human peripheral nerve explants differentially influence macrophage neurotrophic function. CD40 receptor is expressed by macrophages (van Kooten, 2000; van Kooten and Banchereau, 1997) and plays a major role in the regulation of the immune response. The expression of CD40L by infiltrating T-cells during autoimmune disorders, endothelial cells lining cerebral microvessels (Mach et al., 1997) and astrocytes (Calingasan et al., 2002) suggest a broad range of physiologically relevant functions for CD40L in the CNS. Explants of human peripheral nerve and myelin proteins have been shown to augment immune cell mediated neurotrophic activities (Hauben et al., 2000; Schwartz and Moalem, 2001).

We now show that monocyte-derived macrophage (MDM)-conditioned media and MDM-conditioned media from CD40L- and, significantly, from PN-stimulated macrophages increase neuronal survival, synthesis of proteins associated with neurite outgrowth, activity of mitogen-activating protein kinases, ERK1 and ERK2, and synaptic transmission. Production of BDNF and other neurotrophins was associated with these neuronal responses. All together, enhancement of human macrophage neurotrophic and regenerative function by specific environmental cues may play an important role in recovery from CNS injury. Such recovery may serve, in part, to affect the tempo of neurodegenerative diseases.

## 2. Materials and methods

### 2.1. Isolation and propagation of human MDM

Monocytes were obtained from leukopheresis of seven HIV-1, -2 and hepatitis B seronegative donors, and purified by counter current centrifugal elutriation (Gendelman et al., 1988). Wright-stained cytospins prepared from cell suspensions were immunostained with antibodies to CD68 (Dako, cl1KP-1, M0814) and shown to be >98% pure. Monocytes were cultured in Dulbeccos' Modified Eagles Media (DMEM; Sigma Aldrich, St. Louis, MO) with 10% heat-inactivated pooled human serum, 1% glutamine, 50 μg/ml gentamicin (Sigma Aldrich), 10 μg/ml ciprofloxacin (Sigma Aldrich), and 1000 U/ml highly purified recombinant human macrophage colony stimulating factor (MCSF) (a generous gift from Genetics Institute, Cambridge, MA). Monocytes were plated at a density of $2 \times 10^6$ cells/ml for 7 days in Teflon flasks to permit cellular differentiation. Tissue culture reagents were screened before use and found negative for endotoxin (<10 pg/ml; Associates of Cape Cod, Woods Hole, MA) and mycoplasma contamination (Gen-probe II; Gen-probe, San Diego, CA).

### 2.2. Activation of MDM and preparation of MDM-conditioned media

#### 2.2.1. Activation of MDM

MDM were plated at $2 \times 10^6$ cells/ml of DMEM-supplemented media (described above) in Teflon flasks. MDM were stimulated for 18–24 h with either 1 $mm^3$ segment of human peripheral nerve, 1 μg/ml CD40L (a generous gift from Immunex, Seattle, WA) or both. Replicate cells were left untreated. Peripheral nerve from the brachial plexus was collected during the autopsy of four adult patients. The tissue was collected on ice, immediately washed in sterile

PBS, cut into 1 $mm^3$ pieces, and frozen at −80 °C for later use.

#### 2.2.2. Preparation of MDM-conditioned media

Peripheral nerve tissue was removed from flasks immediately following activation. Unstimulated and stimulated MDM were centrifuged at 1200 rpm for 5 min to collect the MDM prior to washing protocol. In order to ensure that residual PN-derived constituents and CD40L were removed from stimulated cells, MDM were washed three times with PBS followed by centrifugation (1200 rpm, as above) after each wash. After the final wash and centrifugation, activated or unstimulated MDM were resuspended in neurobasal media containing 0.5 mM glutamine, 25 μm/ml penicillin and streptomycin (GIBCO-BRL/Life Technologies, Rockville, MD) at $4 \times 10^6$ cells/ml in Teflon 15-ml tubes for 24 h. Parallel cultures were treated with TRIzol for RNA extraction. MDM-conditioned neurobasal media was collected and used fresh or stored at −80 °C.

### 2.3. Neuronal isolation, cultivation and differentiation

#### 2.3.1. Isolation and cultivation of rat cortical neurons

Cerebral cortex was dissected from day 17 embryonic Sprague–Dawley rats (Sasco, Wilmington, MA), mechanically dissociated in $Ca^{2+}$/$Mg^{2+}$-free Hank's balanced salt solution (HBSS), with 0.035% sodium bicarbonate and 1 mM pyruvate (pH 7.4) following 20 min digestion with 0.1% trypsin. Trypsin was neutralized with 10% fetal bovine serum and the cell suspension was washed three times in HBSS. Cells were resuspended and cultured in neurobasal media supplemented with B27, and plated onto poly-D-lysine-coated plates (Sigma, St. Louis, MO). Over 95% of cells stained for neuronal markers after the first day of culture and greater than 80% stained for neuronal antigens after 2 weeks. Cells were plated onto poly-D-lysine-coated dishes at densities indicated below. One day after plating, cells were treated with MDM-conditioned media or neurobasal media with or without NGF (100 ng/ml, GIBCO-BRL/Life Technologies) and epithelial growth factor (EGF, 50 ng/ml, GIBCO-BRL/Life Technologies).

#### 2.3.2. Rat cortical neuron viability measurements

Viability of rat cortical neurons was measured by metabolism of thiazolyl blue, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich). Rat cortical neurons were plated at $5 \times 10^4$ cells/well in poly-D-lysine-coated 96-well plates in NBM with B27 supplement. After 24 h, cortical cultures were rinsed in NBM without B27 and treated with MDM-conditioned media or neurobasal media with or without NGF and EGF for 5 days in vitro (DIV). Viability was assessed by measurements of mitochondrial activity. Ten microliters of MTT substrate was incubated with cultures for 60 min. Thirty microliters of dimethylsulfoxide was added to cultures, rocked at room temperature for 15 min and absorbance read at 490 nm on a Vmax plate reader (Molecular Devices, Sunnyvale CA).

#### 2.3.3. Survival and differentiation of rat cortical neurons

Quantitative enzyme-linked immuno-sorbent assays (ELISA) were used to determine the presence of surviving and differentiating rat cortical neurons as shown by Zheng et al. (2001). Briefly, rat cortical neurons were plated in 96-well plates at $5 \times 10^4$ cells/well, treated with MDM-conditioned media or neurobasal media and cultured for 5–6 DIV. After treatment, cultures were fixed with 4% paraformaldehyde in PBS. Cortical cultures were washed three times with PBS, and treated with 3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase. Cortical cultures were treated with 3% normal goat serum (NGS) in PBS for 10 min at room temperature and incubated with antibodies against MAP-2, or Tau in 3% NGS for 1 h. Cortical cultures were washed three times with PBS and incubated with mouse or rabbit biotinylated antibodies (Vector Laboratories, Burlingame, CA) in 3% NGS for 30 min at room temperature. After three additional washes with PBS, avidin–biotin complex solution (that forms the avidin–biotinylated enzyme complex; Vector Laboratories) was added for 30 min at RT. Color was developed using the substrate 3,3′,5,5′ tetramethylbenzidine (Vector Laboratories) for 4 min and reaction was terminated with 1 M $H_2SO_4$ (Sigma). The absorbance was read at 450 nm on a Vmax plate reader (Molecular Devices). Serial dilutions of neurons were assayed to generate a standard curve (Zheng et al., 2001). Quadruplicate values for optical density for MAP-2 antigen generated a linear standard curve, $y = 84.982x - 89.121$, $R^2 = 0.9628$. Quadruplicate values for optical density for Tau antigen generated an exponential standard curve, $y = 2.678e^{1.7061x}$, $R^2 = 0.9813$.

### 2.4. MAP kinase (MAPK) measurements

Rat cortical neurons were cultured on 24-well poly-D-lysine-coated culture dishes ($5 \times 10^5$ cells/well) for 1 day in neurobasal media containing B27. Cortical neurons were deprived of serum or B27 24 h after plating, then stimulated with NGF plus 50 ng/ml EGF or MDM-conditioned media for 5 min. Cells were lysed in 0.5% Triton lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 0.5% Triton, 0.2% SDS, 1% BSA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, and 1 mM PMSF). Clarified cell lysates were immunoprecipitated using antibodies to Erk1 and Erk2 (Erk1/2). Erk 1/2 MAP kinase assays were performed using myelin basic protein as a substrate (Rao et al., 1994). Reactions were stopped by addition of SDS sample buffer and heating the samples to 100 °C for 3 to 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 12% gel. Radiolabeled myelin basic protein was quantified using phosphor storage (Molecular Dynamics, Sunnyvale, CA). Parallel experiments were performed with

cortical cultures pretreated for 30 min with the MAP kinase inhibitor PD98059 (30 μM, GIBCO-BRL/Life Technologies). To determine the effect of MAPK pathway activation on neuronal survival and dendritic differentiation, cortical cultures in 96-well plates were treated with MDM and control media in the presence of the MAPK pathway inhibitor PD98059 (30 μM; GIBCO-BRL/Life Technologies) for 6 DIV. MAP-2 ELISA was performed and analyzed as described above.

### 2.5. Assay for synthesis of MAP-2 in rat cortical neurons

Rat cortical neurons were cultured on 24-well poly-D-lysine-coated culture dishes ($5 \times 10^5$ cells/well) for 4 to 6 days in neurobasal media containing B27. Cultures were washed twice in neurobasal media without B27 and treated for 12 h with control, CD40L, PN and CD40L/PN MDM-conditioned media in 5% $CO_2$ at 37 °C. Neurobasal media and neurobasal media plus NGF and EGF were used as negative and positive controls. Cells were rinsed twice and cultured in DMEM without methionine and cysteine for 1 h. Cells were cultured for 4 h in DMEM without methionine and cysteine plus 75 μCi per well of Easy Tag Express Methionine/Cysteine protein labeling Mix $^{35}S$ (NEN Life Science Products, Boston, MA). Cells were rinsed with 4 °C PBS and flash frozen in liquid nitrogen. Cells were lysed with Igepal lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2 mM DTT, 2% Igepal, 0.2% SDS, 1% BSA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, and 1 mM PMSF) and centrifuged for 10 min at 15,000 rpm. Supernatants were incubated with 5 μl of MAP-2a,b primary antibody (Sigma Aldrich) overnight at 4 °C. Protein G beads immunoprecipitated the proteins from lysates. Beads were washed three times in lysis buffer. Collected beads were treated with 2× SDS sample buffer and heated for 5 min at 95 °C. Samples were run on a 4–20% SDS denaturing gel. Radiolabeled MAP-2a,b was quantified using phosphor storage (Molecular Dynamics).

### 2.6. Hippocampal slices and electrophysiological recordings

Hippocampal brain slices were dissected from 15- to 35-day-old male Sprague–Dawley rats. Brains were quickly removed from the cranial cavity and placed into an ice-cold (4 °C) oxygenated artificial cerebrospinal fluid (ACSF) environment. Hippocampi were dissected and brain tissue slices (400 μm in thickness) were cut transversely using a tissue chopper. Slices were kept in a humidified/oxygenated holding chamber at room temperature for at least 1 h before being transferred to a recording chamber. In the recording chamber, single hippocampal slices were submerged and continuously perfused in ACSF (124 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM $NaH_2PO_3$, 26 mM $NaHCO_3$, and 10 mM glucose; equilibrated with 95% $O_2$ and 5% $CO_2$; pH 7.35–7.45) at a constant flow rate of 2 ml/min using a peristaltic pump (Rainin Instrument, Woburn, MA). The temperature of the perfusate was maintained at 30 °C with an automatic temperature controller (Warner Instrument, Hamden, CT). MCM were applied to hippocampal slices at a dilution of 1:10 with ACSF using a peristaltic pump. Diluted supernatants were equilibrated with 95% $O_2$ and 5% $CO_2$ for 5 to 10 min before and during bath application. Excitatory postsynaptic potentials (EPSPs) were elicited by a constant current stimulation (0.05 Hz, 50–400 μA) of Schaffer collateral-commissural axons using an insulated bipolar tungsten electrode. Intensity and duration of stimulation were adjusted to generate approximately 30–40% of a maximal response. Evoked EPSPs were recorded with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA) in the CA1 dendrite field (stratum radium). Recordings were made with borosilicated glass microelectrodes with a tip diameter of 2.5–5.0 μm and a resistance of 1–5 MΩ when filled with ACSF. A 10–15-min control recording was conducted in each experiment once stimulation parameters were achieved. Each recording was an average of three consecutive evoked signals. Initial slopes of field EPSPs were measured and expressed as a percent of basal levels (before application of MDM-conditioned media). Values reflected the actual voltage amplitude. Signals were digitized at a frequency of 5 kHz using a Digidata 1200 interface (Axon Instruments) and data stored with a desktop computer.

### 2.7. Western blots

Rat cortical neurons cultured on 24-well poly-D-lysine-coated culture dishes ($5 \times 10^5$ cells/well) for 1 day were then washed and treated with control or stimulated MCM. After 6 days, cells were lysed with Igepal lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2 mM DTT, 1% Igepal, 0.2% sodium dodecyl sulfate (SDS), 1% BSA, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerolphosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, and 1 mM PMSF]. Lysates were clarified and amount of total protein determined. Total protein was run from each experimental and control sample. Thirty-five micrograms of total protein was run for each sample as determined by the Bradford method (BioRad, Hercules, CA). After electrophoretic transfer to polyvinyldifluoridene (PVDF) membranes, proteins were treated with primary antibodies to neuron-specific nuclear protein (NeuN, Chemicon International, Temecula, CA) and GFAP (Sigma Aldrich) overnight at 4 °C. Membranes were washed, incubated with secondary antibody conjugated to alkaline phosphatase and visualized with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim, Indianapolis, IN). Images were digitized using a Molecular Dynamics densitometer (Molecular Dynamics).

### 2.8. Cellular assays for determination of primary MDM neurotrophic properties

#### 2.8.1. Cultivation of PC12 cells

Rat pheochromocytoma (PC12) cells were purchased from American Type Culture Collection (ATCC), and maintained in Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum (Sigma Aldrich). Cells were passaged in 75-mm tissue culture flasks.

#### 2.8.2. PC12 cell differentiation

PC12 were plated on collagen-coated 24-well culture plates at $5 \times 10^4$ cells/well in neurobasal media plus 10% fetal bovine serum. After 1 day in vitro, cells were washed and treated with neurobasal media plus NGF (20 or 100 ng/ml, GIBCO-BRL/Life Technologies) and MDM-conditioned media plus NGF (20 ng/ml). Cells were incubated at 37 °C for 72 h then assessed for cellular differentiation compared to untreated controls. Differentiation was determined by counting the numbers of cells found extending neurite-like processes twice the length of the soma out of 100 total cells counted. Differentiation of PC12 cells by NGF has been well defined (Greene and Tischler, 1976) and frequently used to study issues of neuronal differentiation and intracellular signaling (Vaudry et al., 2002).

### 2.9. Real-time quantitative RT-PCR

Total cellular RNA was isolated using TRIzol (Life Technologies). Cells were lysed and RNA extracted using chloroform (Chomczynski, 1993). Levels of BDNF, IL-6, IL-1β, IL-10 expression was determined by RT-PCR. RNA samples were reverse-transcribed with random primers and the cDNA was used for real-time quantitative PCR. One microgram of RNA was reverse transcribed with Superscript II reverse transciptase (Invitrogen) using random hexamers (2.5 μM), dNTPs (500 μM each) in first-strand buffer. The reaction was performed at 25 °C for 10 min followed by 30 min at 48 °C and 5 min at 95 °C. Though the RNA samples were treated with RNase-free Dnase, -RT controls were ran simultaneously to see possible genomic DNA contamination. Real-time quantitative PCR was performed using the ABI prism 7000 Sequence detector (Perkin Elmer, CA). The primers were designed using Primer express software supplied with the instrument and are listed in Table 1. SYBR green I was used in the PCR reaction. SYBR green specifically binds to double-stranded DNA and only emits fluorescence when bound to double-stranded DNA. The 50-μl PCR reaction mix consisted of SYBR green PCR buffer, $MgCl_2$ (3 mM), dNTPs with dUTP (50 μM each), Ampli Taq Gold (0.625 U), AmpErase UNG (0.25 U) and the reaction was carried out in a 96-well plate at the thermal cycling conditions − 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primer concentrations were optimized for each set. All reagents were obtained from Applied Biosystems, CA. The reactions were run on a dissociation protocol to generate a Tm curve of the product formed. Gene expression of different cytokines was calculated using 18s ribosomal RNA as an endogenous control. The relative standard curve method (User Bulletin #2, ABI Prism 7000 sequence detection system) was used to generate standard curves that determined amplification efficiency of each gene, from which the normalized expression of each gene was calculated relative to the endogenous control.

Table 1
Oligonucleotides used for RT-PCR

| Target | Primer sequences | Amplicon size |
|---|---|---|
| BDNF | sense 5′CGGGCCCTTACCATGGATA3′<br>antisense 5′CCTTATGAATCGCCAGCCA3′ | 51 |
| IL-10 | sense 5′CTACGGCGCTGTCATCGAT3′<br>antisense 5′CACGGCCTTGCTCTTGTTTT3′ | 51 |
| IL-6 | sense 5′GTGGCTGCAGGACATGACAA3′<br>antisense 5′TGAGGTGCCCATGCTACATTT3′ | 101 |
| IL-1β | sense 5′CAGGGACAGGATATGGAGCAA3′<br>antisense 5′TTTTCCTTGAGGCCCAAGG3′ | 101 |

### 2.10. Neurotrophin detection

#### 2.10.1. ELISA

MDM-conditioned medias were analyzed by ELISA for IL-6, NGF, BDNF, and NT-3 (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Levels of neurotrophins were normalized to cell number based on MTT assays.

#### 2.10.2. Surface enhanced laser desorption ionization (SELDI) ProteinChip®

MDM-conditioned medias were collected from control and PN-stimulated MDM (for 24 h). Low molecular weight proteins were concentrated by filtration using Centricon centrifugal filtration (Millipore, Chicago, IL) with a 50-kDa molecular weight cutoff. Elutates were collected and the total protein concentration measured with the Pierce BCA assay (Pierce, Pittsburgh, PA). Samples were diluted to a final concentration of 0.5 μg/μl in sterile PBS. An H4 (aliphatic hydrophobic) ProteinChip® array (Ciphergen, Fremont, CA) was used for protein profiling. One microgram of total protein was dried onto a single spot on the chip. Spots were covered with 0.5 μl of saturated sinapinic acid (SPA) energy-absorbing matrix (EAM) prepared in an aqueous solution containing 50% acetonitrile and 0.5% trifluoroacetic acid and then allowed to air dry. Data were collected using both low and high laser intensities with the ProteinChip® reader previously calibrated with a broad range of internal standards (Bovine Ubiquitin, Equine Myoglobin, Horse Radish Peroxidase and Chicken Conalbumin A). These were used to normalize data and calibrate peak mass, making comparisons possible.

For antibody capture tests, monoclonal antibody to BDNF (R&D Systems) was reconstituted as 0.5 mg/ml stock in PBS, aliquoted and then stored frozen. To a PS1

(preactivated/biological surface) ProteinChip®, 2 μl of antibody was added to each spot and incubated in a humidity chamber for 2 h at room temperature. Residual active sites were blocked by incubation with 5 μl/spot of 1 M ethanolamine (pH 8) in PBS for 20 min in a humidity chamber. Protein spots were washed 2× with 0.5% Triton X-100 in 1× PBS for 15 min and three times with PBS for 5 min. Following washes, 2.5 μg of sample or 400 ng of recombinant peptide was added to the spot. Chips were incubated in a humidity chamber for 2 h, washed with 0.5% Triton X-100 and PBS, and rinsed with HPLC-grade water. Chips were dried, covered with 0.5 μl of SPA and dried again. Data were collected and concentrations of BDNF were calculated for each sample using amounts determined from recombinant standards.

### 2.11. Statistical analyses

Data were analyzed using Graphpad Prism 3.0 software with one-way ANOVA used to test differences between groups, between activation paradigms, and neuronal culture conditions. If the overall tests were significant, pair-wise comparisons were conducted to determine differences. ANOVA was performed with Newman–Keuls post-test (for multiple comparisons) unless otherwise specified.

## 3. Results

### 3.1. Macrophage-conditioned media increased mitochondrial activity, neuronal survival and neurite outgrowth

Neuronal viability was determined by mitochondrial activity of neurons treated with neurobasal media or neurobasal media containing 100 ng/ml NGF and 50 ng/ml EGF. Addition of growth factors NGF and EGF to neurobasal culture media increased mitochondrial activity of cortical neurons 2-fold above treatment with neurobasal media alone. Control and stimulated MDM-conditioned media significantly increased mitochondrial activity of cortical neurons when compared to neurobasal media and neurobasal media plus NGF and EGF (Fig. 1A, $n=4$, $p<0.001$). PN-, CD40L- and CD40L plus PN–MDM-conditioned media all equally increased mitochondrial activity in rat cortical neurons ($p>0.05$, Fig. 1A).

To determine the specific effect of MDM secretory products on neuronal survival and neurite outgrowth in cortical cultures, ELISA assays for MAP-2 and Tau neuronal antigens were performed (Fig. 1B,C). MAP-2 is expressed primarily in soma, newly extending processes and mature dendrites while Tau is expressed in differentiated axons (Aletta et al., 1988). Quantitative analysis of MAP-2 and Tau expressing cells and processes was determined and compared to standard curves generated by serial dilutions of immunostained cells (see Materials and methods).

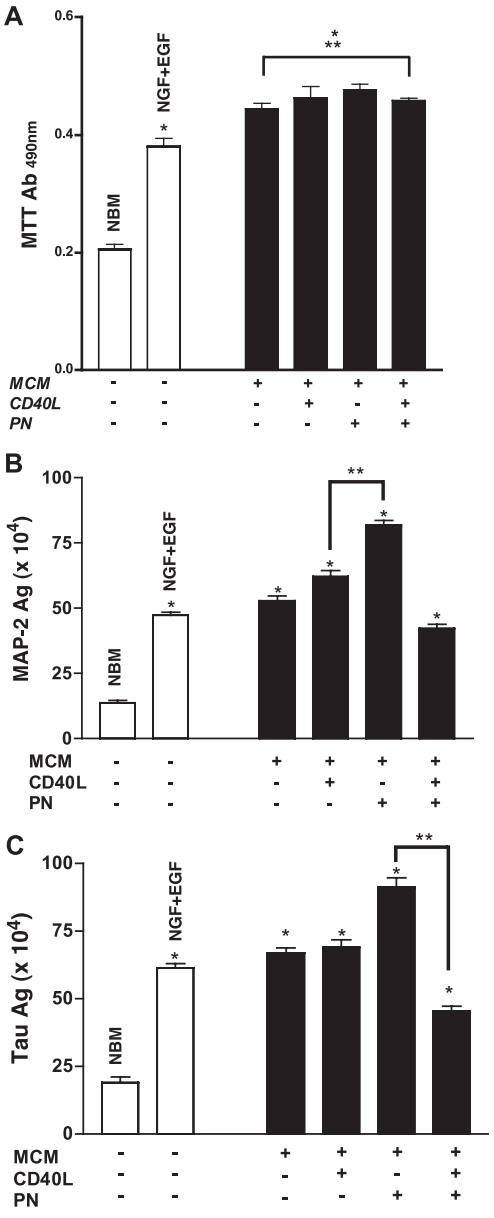


Fig. 1. Monocyte-derived macrophage (MDM)-conditioned media increases neuronal survival and neurite outgrowth. (A) Mitochondrial activity of cortical cultures was assessed by MTT assays. MDM-conditioned media (MCM) were placed on primary rat cortical neurons for 5 days. Results shown are the average ± S.E.M. of normalized values from four separate experiments (with four different donors). (B) Neuronal viability and extension of dendrites was assessed by MAP-2 and (C) by the Tau ELISA. Replicate cultures were treated with one of the following: neurobasal media (NBM), neurobasal media plus 100 ng/ml NGF and 50 ng/ml EGF (NBM plus NGF + EGF), or conditioned media from either unstimulated MDM (MCM control), CD40L (CD40L MCM), peripheral nerve (PN MCM), or CD40L plus peripheral nerve stimulated MDM (CD40L + PN MCM). Data are expressed as means ± standard deviation (S.D.). *Indicates a $p<0.001$ when compared to neurobasal media (NBM). **Indicates a $p<0.001$ when compared to NBM plus NGF and EGF. All treatments were performed in replicates of four from four different donors. MCM is MDM-conditioned media and NBM is Neurobasal media.

Neurobasal media plus NGF and EGF increased the MAP-2 antigen expression 71% above neurobasal media alone ($p < 0.001$). Conditioned media from unstimulated (control) MDM significantly increased MAP-2 antigen expression above neurobasal media (73%, $p < 0.001$) but not neurobasal media plus NGF and EGF (10%, $p > 0.05$, Fig. 1B). Stimulation of MDM with CD40L increased MAP-2 antigen expression compared to neurobasal media (78%, $p < 0.001$) and neurobasal media plus NGF and EGF (24%, $p < 0.001$, Fig. 1B). However, PN–MDM-conditioned media showed the highest increase in MAP-2 antigen expression when compared to neurobasal media (83%, $p < 0.001$, Fig. 1B) and compared to neurobasal media plus NGF and EGF (43%, $p < 0.001$, Fig. 1B). CD40L plus PN–MDM-conditioned media significantly increased MAP-2 antigen expression compared to neurobasal media but at lower levels

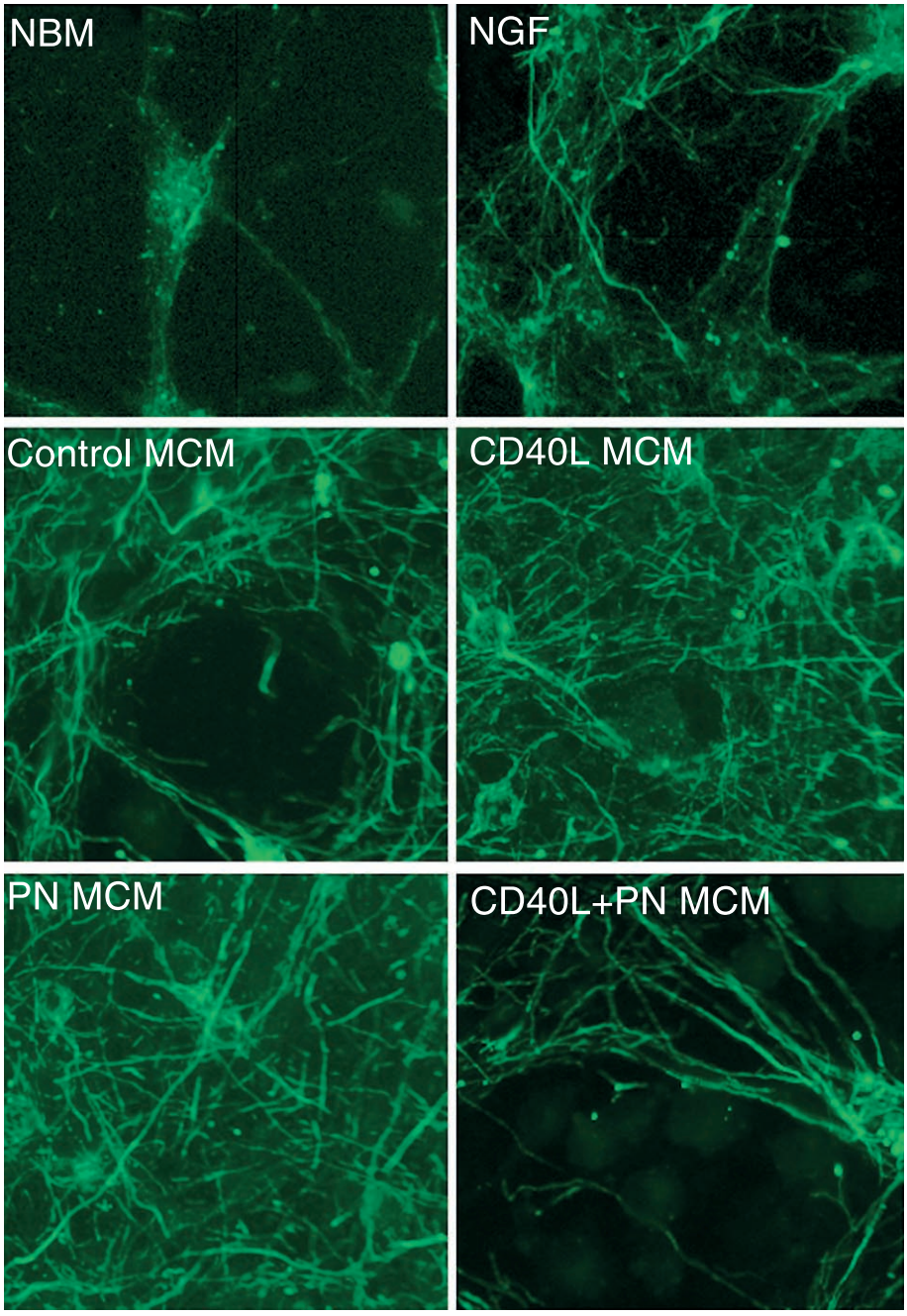


Fig. 2. Neurite outgrowth is more extensive in primary rat cortical neurons following treatment with MCM. Twenty-four hours after rat cortical neurons were plated, cortical cultures were treated with control or stimulated MCM for a period of 6 DIV. Cells were fixed with acetone/methanol and stained with anti-neurofilament antibodies. Each panel indicates the specific treatment in the upper left-hand corner. Unstimulated MDM medias are labeled control, stimulated medias are labeled according to the particular cue used to activate MDM. MCM is MDM-conditioned media.

(68%, $p < 0.001$). Overall, significantly decreased MAP-2 antigen expression was seen in rat cortical neurons treated with CD40L plus PN–MDM-conditioned media compared to the MAP-2 antigen expression seen in control, CD40L– or PN–MDM-conditioned media treated groups ($p < 0.001$, Fig. 1B). Tau expression was also increased following treatment of cortical cultures with MDM-conditioned media. Neurobasal media alone did not adequately support the extension of axonal processes (Fig. 1C). A 69% increase in Tau expression was observed in cortical neurons treated with neurobasal media plus NGF and EGF (Fig. 1C). While all MDM-conditioned media significantly increased Tau expression above neurobasal media ($p < 0.001$), PN–MDM-conditioned media exhibited the most dramatic effects (79%, Fig. 1C). Only PN–MDM-conditioned media significantly increased Tau expression by 33% compared to neurobasal media plus NGF and EGF ($p < 0.001$, Fig. 1C). CD40L plus PN–MDM-conditioned media caused a significant *decrease* in Tau expression compared to neurobasal media and growth factor supplemented neurobasal media.

To determine whether MDM-conditioned media promote the extension of processes and differentiation of neurons we treated primary rat cortical neurons with MDM-conditioned media and assessed total neurite outgrowth. Following treatment with control and MDM-conditioned media for 5–6 days in vitro (DIV) neuronal cultures were fixed in acetone/methanol and stained with anti-neurofilament antibody. Fig. 2 shows the morphology of both axons and dendrites of cortical neurons following treatment with control and MDM-conditioned media. Neuronal processes cultured in neurobasal media alone appeared tightly fasciculated. Neurobasal media supplemented with NGF supported process outgrowth. Neurobasal media conditioned

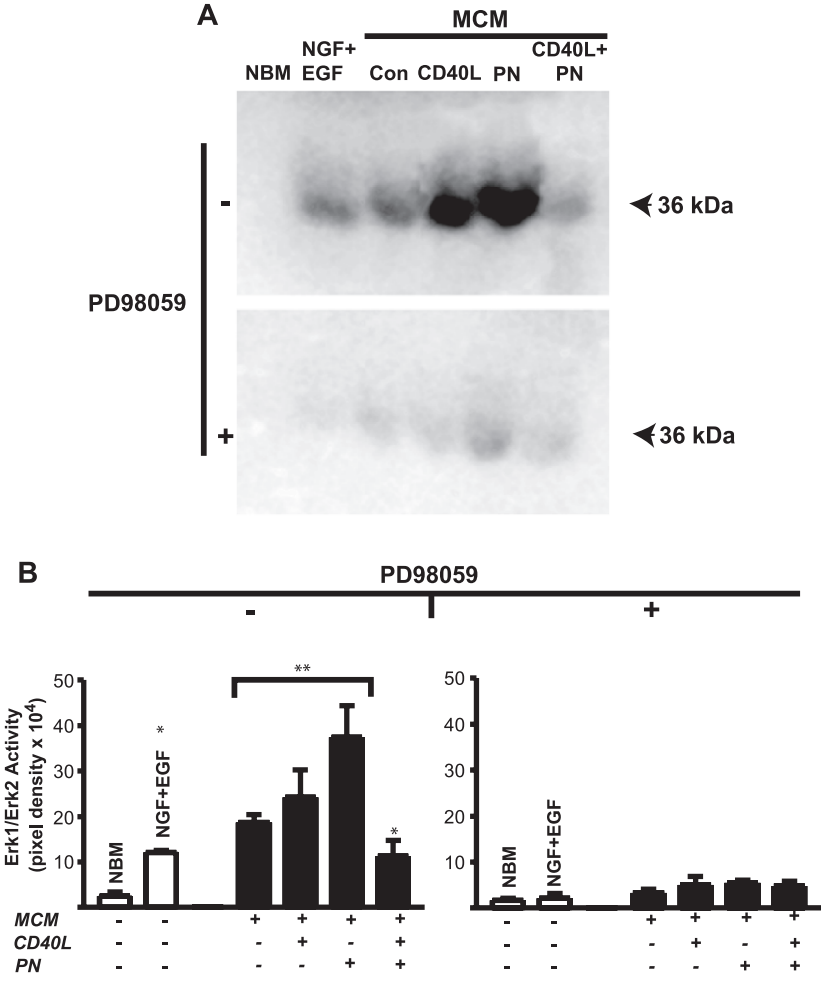


Fig. 3. MAPK activation in rat cortical cultures following exposure to MCM. ERK 1/2 activation was assessed in rat cortical cultures following application of MCM. Activation of ERK 1/2 was determined by their ability to phosphorylate myelin basic protein (MBP, MW of dimer is 36 kDa). PN-MCM media elicited the most dramatic increase in ERK 1/2 activation (A). Increased ERK 1/2 activation was blocked by the specific MAPK inhibitor, PD98059 (30 μm, A). Radioactivity was analyzed from two independent experiments using the Phospho Plus imaging system and these data are shown in (B). Error bars represent S.E.M. from two independent experiments done in triplicate. *Indicates significance of $p < 0.001$ when compared to neurobasal media (NBM) control. **Indicates a $p < 0.001$ when compared to neurobasal media plus NGF and EGF (NBM + NGF and EGF). MCM is MDM-conditioned media.

with unstimulated MDM promoted neurite outgrowth to a greater extent than NGF supplemented neurobasal media. PN–MDM-conditioned media and CD40L–MDM-conditioned media, induced an increased extension and development of neuronal processes. CD40L plus PN–MDM-conditioned media induced less dramatic process outgrowth and caused an increase in neuronal fasciculation. Taken together, these data show that MDM promote neuronal survival, process extension and neuronal differentiation of primary cortical neurons and that this endogenous neurotrophic effect can be enhanced by pre-activation with peripheral nerve and to a lesser extent CD40L.

### *3.2. MCM activated the MAPK signal transduction pathway in rat cortical neurons*

Growth factor mediated differentiation and neurite outgrowth of CNS neurons is associated with activation of MAPK (Marshall, 1995). To determine whether neurite outgrowth induced by MDM-conditioned media is associated with stimulation of MAPK, we measured the activity of ERK 1/2 using an in vitro kinase assay. Endogenous ERK 1/2 were immunoprecipitated from cell lysates and the kinase assay was performed with myelin basic protein (MBP) and [$\gamma$-$^{32}$P] ATP as co-substrates. Evaluation of MBP and [$\gamma$-$^{32}$P] ATP levels showed that treatment with growth factors NGF (100 ng/ml) and EGF (50 ng/ml) increased ERK 1/2 significantly above cellular resting levels in neurobasal media ($p<0.01$) (Fig. 3A and B). Control MDM-conditioned media, CD40L-, and PN–MDM-conditioned media significantly increased ERK 1/2 above that observed following treatment with neurobasal media plus NGF and EGF ($p<0.01$). CD40L plus PN–MDM-conditioned media caused significantly less ERK 1/2 activation than MDM-conditioned media, CD40L–MDM-conditioned media, or PN–MDM-conditioned media ($p<0.01$) but this was not significantly different than growth factor stimulation ($p>0.05$). All of the MDM-conditioned media increased MAPK pathway activity as compared to basal activity. As illustrated in Fig. 3, PN–MDM-conditioned media showed the greatest increase in ERK 1/2 activity (15-fold above) compared to basal levels ($n=3$, $p<0.01$). On average, CD40L increased ERK 1/2 nearly 10-fold above basal levels and CD40L plus PN–MDM-conditioned media increased ERK 1/2 4.5-fold above basal levels. The MAPK-specific inhibitor PD98059 (30 μm) blocked MAPK activation indicating the specificity of ERK 1/2 responses by MDM-conditioned media (Fig. 3B). Further, treatment of cortical neuron cultures with PD98059 (30 μm) during their incubation with MDM-conditioned media dramatically reduced the number of neurons expressing MAP-2 antigen in neurobasal media plus NGF and EGF as well as MDM-conditioned media treated cultures (Fig. 3B). PN–MDM-conditioned media treated cortical neurons were the only group to show a significant increase in MAP-2 antigen expression ($p<0.001$).

### *3.3. Macrophage-conditioned media increased synthesis of MAP-2a,b*

Activation of the MAPK pathway is often associated with an upregulation of protein synthesis. To determine whether MDM-conditioned media stimulates the synthesis of new proteins required for outgrowth or regeneration of neuronal processes, we assessed the synthesis of MAP-2 following exposure to MDM-conditioned media. A representative radiolabeled Western blot illustrates that neurobasal media containing NGF (100 ng/ml) and EGF (50 ng/ml) increased MAP-2 synthesis >2-fold when compared to neurobasal media alone (Fig. 4A and B). Neurons exposed to MDM-conditioned media increased the synthesis of MAP-2 2-fold above neurobasal media (Fig. 4A and B). Neurons treated with CD40L–MDM-conditioned media minimally increased MAP-2 when compared to control MDM-conditioned media (Fig. 4A and B). PN–MDM-

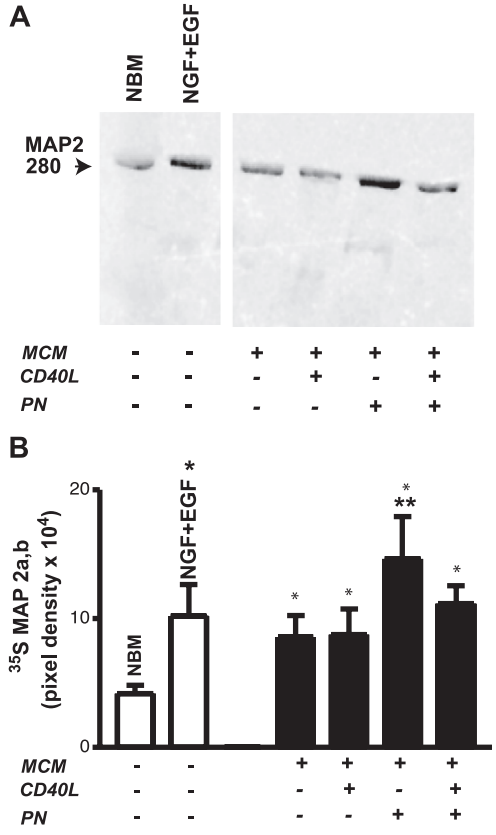


Fig. 4. Neuronal protein synthesis following exposure MCM. Methionine-starved rat cortical neurons (for 4–5 days) were stimulated with MCM for 4 h and then labeled with $^{35}$S-methionine. Following labeling, cells were lysed using a 1% Igepal (Sigma) lysis buffer. Lysates were immunoprecipitated with a monoclonal anti-MAP2a+b antibody over night. Bound MAP2a+b proteins (MW, 280 kDa) were separated on 4–20% SDS-PAGE gel (Biradford method, Biorad). Radioactivity was analyzed using the Phospho Plus imaging system. Data represent the results of three separate experiments. *Indicates a $p<0.001$ when compared to neurobasal media (NBM). **Indicates a $p<0.001$ when compared to neurobasal plus NGF and EGF (NBM+NGF and EGF). MCM is MDM-conditioned media and NBM is neurobasal media.

conditioned media induced the largest increase in MAP-2 protein synthesis causing nearly a 3.5-fold increase over neurobasal media (Fig. 4A and B). CD40L plus PN–MDM-conditioned media stimulated MAP-2 ~ 3-fold above neurobasal media (Fig. 4A and B). These results show that culturing rat cortical neurons in the presence of MDM-conditioned media, and in particular, PN–MDM-conditioned media increased the synthesis of MAP-2, a microtubule associated protein necessary for neurite outgrowth in primary cortical neurons (Diaz-Nido et al., 1990; Ferreira et al., 1987; Sharma et al., 1994).

### 3.4. Macrophage-conditioned media directly promotes neuronal survival and neurite outgrowth

The ability of macrophages to activate glial cells has been well documented (Minagar et al., 2002; Raivich et al., 1996). In order to determine whether MDM-conditioned media increased astrocyte survival, proliferation or activation in primary cortical cultures, glial fibrillary acidic protein (GFAP) and protein content was assessed following treatment of cortical cultures with conditioned media. Quantification of the Western blots shows that control, PN-, or CD40L–MDM-conditioned media increase GFAP >5-fold that of NBM (Fig. 5). These results suggest that astrocytes are indeed regulated by MDM-conditioned media. Consequently, astrocytes may play a role in the MDM neurotrophic properties observed in our cellular assays. In order to determine whether MDM secretory products are neurotrophic independent of astrocyte effects, we examined whether MDM-conditioned media support the survival and neurite outgrowth of the well-characterized rat phochromocytoma cell line (PC12). PC12 cells differentiate into neuron-like cells following treatment with NGF as well as other neurotrophins (Greene and Tischler, 1976; Vaudry et al., 2002). Treatment of PC12 cells with 100 ng/ml of NGF promotes the differentiation of nearly 100% of cultured PC12 cells over several weeks in culture. Sub-optimal doses of NGF do not promote a similar rate of differentiation (Greene and Tischler, 1976).

Using this cellular assay system, we compared neurite outgrowth in PC12 cells treated with an optimal dose of NGF (100 ng/ml), a sub-optimal dose of NGF (20 ng/ml), and MDM-conditioned media following the removal of sub-optimal dose of NGF (20 ng/ml). After 2 days, in culture the numbers of differentiating cells (those extending processes at least twice the length of the cell's soma) were counted. We assessed this very early time point to clearly identify the ability of secreted factors to initiate neurite outgrowth. MDM-conditioned media significantly increased the ability of PC12 cells to differentiate in the presence of 20 ng/ml NGF (Fig. 6A,B). Control MDM-conditioned media enhanced neurite outgrowth and differentiation (11.9 ± 1.3%) similar to that of media supplemented with a maximum dose of 100 ng/ml NGF (11.2 ± 2.4%). PN–MDM-conditioned media significantly increased the number of cells extending processes to 24.9 ± 3.9% above the control numbers at 2.6 ± 1.0% ($p < 0.01$, Fig. 6B). To a lesser extent, CD40L–MDM-conditioned media and CD40L plus PN–MDM-conditioned media increased PC12 cell differentiation above control levels to 14.7 ± 1.5% and 13.3 ± 5.0%, respectively. These data suggest that activated MDM-conditioned media, in particular PN–MDM-conditioned media, directly potentiate neurite outgrowth and differentiation independent of astrocytes.

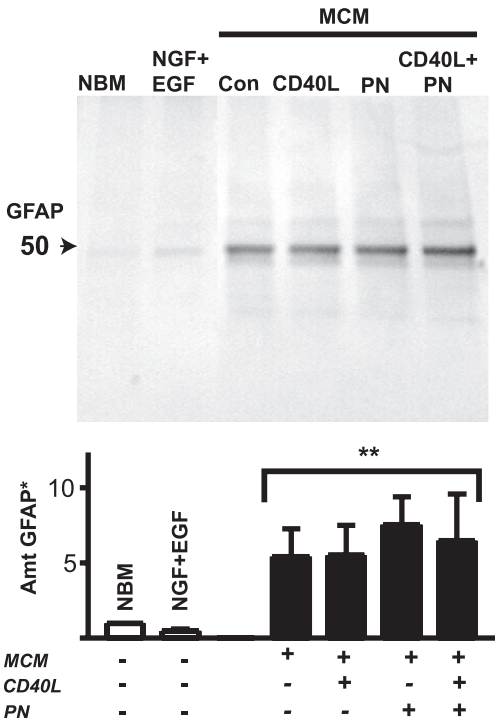


Fig. 5. Western blot analysis of astrocytic proteins following exposure to MCM. Lysates of primary rat cortical neurons treated with neurobasal media (NBM), neurobasal media (NBM) plus NGF and EGF, unstimulated MCM CD40L stimulated MCM (CD40L MCM), PN-MCM and CD40L plus PN stimulated MCM (CD40L + PN MCM) were immunoprecipitated with marker proteins for astrocytes, GFAP. Western blots of these proteins were analyzed for protein content using Gel expert software. The quantitation illustrated below the representative Western blot is the compilation of data measured from three separate experiments. Error bars indicate the S.E.M. for each data set. *Indicates significance of $p < 0.001$ when compared to NBM control. **Indicates a $p < 0.001$ when compared to neurobasal (NBM) plus NGF and EGF. MCM is MDM-conditioned media and NBM is neurobasal media.

### 3.5. Macrophage-conditioned media increased synaptic transmission in hippocampus

We investigated field excitatory postsynaptic potentials (EPSPs) in the CA1 region of rat hippocampal brain slices to determine MDM-conditioned media effects on synaptic transmission. Bath application of PN–MDM-conditioned media showed a significant enhancement of field EPSPs when compared to MDM-conditioned media (Fig. 7A–C). The initial slope of the EPSP measured at peak response was 154.6% of basal levels when the slices were perfused with

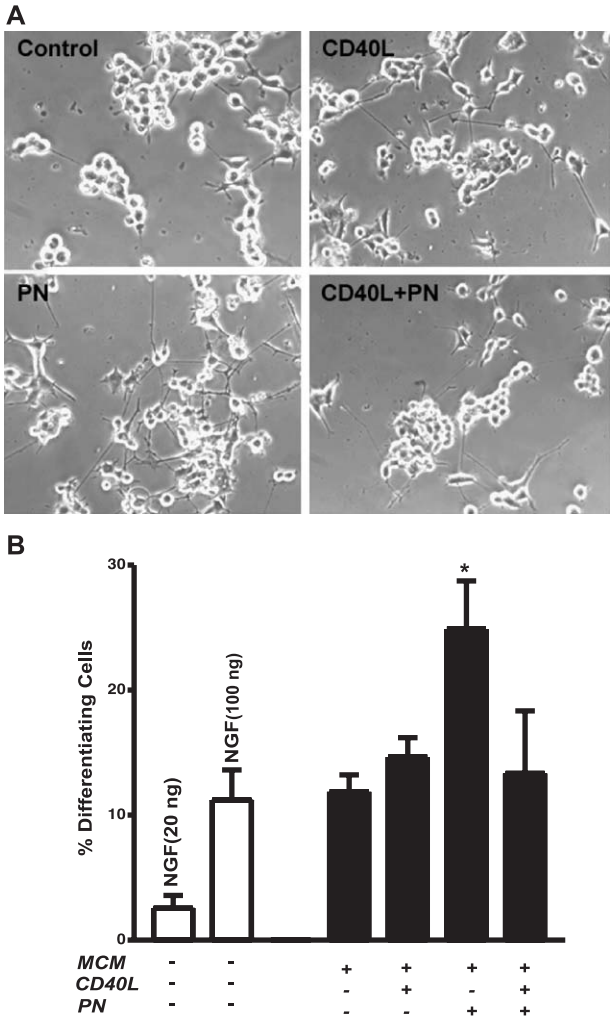


Fig. 6. MDM-conditioned media enhance the differentiation of PC12 cells. Phase contrast photomicrographs show that PC12 cells are stimulated to differentiate following treatment with 20 ng/ml NGF in the presence of MCM (A). Magnification is × 400. Quantitation of differentiation of PC12 cells following priming MCM is shown in (B). Differentiation or process outgrowth in PC12 cells following treatment with 20 ng/ml NGF alone or 100 ng/ml NGF alone or in the presence of MDM-conditioned media was determined by counting the number of cells in each condition that extended processes twice the length of the neuronal cell body. Data are representative of three independent experiments. *Indicates significance of $p < 0.01$ when compared to neurobasal media (NBM) and neurobasal media plus NGF (NBM plus NGF). MCM is MDM-conditioned media.

PN–MDM-conditioned media (Fig. 7A, solid circle). In contrast, bath application of MDM-conditioned media had no apparent effect on synaptic transmission (Fig. 7, open triangle). Evoked EPSPs showed a significant increase following application of PN–MDM-conditioned media while remaining unchanged following application of MDM-conditioned media (Fig. 7B). On average, the initial slope of the EPSPs measured at peak response and following bath application of PN–MDM-conditioned media was 130.0 ± 9.4% ($n = 17$) while MDM-conditioned media was 104.5 ± 4.6% of basal levels ($n = 15$, $p < 0.05$; Fig. 7C). CD40L–MDM-conditioned media increased the EPSP initial slope (measured at response peak) to 119.4 ± 16.5% of basal levels ($n = 14$), although the increase was not statistically significant ($p > 0.05$). The peak EPSPs after bath application of CD40L plus PN–MDM-conditioned media was 109.5 ± 15.1% of basal levels ($n = 16$). This difference was not significant when compared to the peak response obtained from slices treated with MDM-conditioned media ($p > 0.05$, Fig. 7C). To control for any effects of the media used to cultivate the MDM-conditioned media on synaptic function, we assayed EPSP after bath application of neurobasal media. Such applications had no effect on field EPSPs on the hippocampal slices ($n = 28$) (data not shown).

### 3.6. Stimulated macrophages show increased synthesis of neurotrophic proteins

We have shown several lines of evidence indicating that PN- or CD40L-stimulated MDM (and to a lesser extent

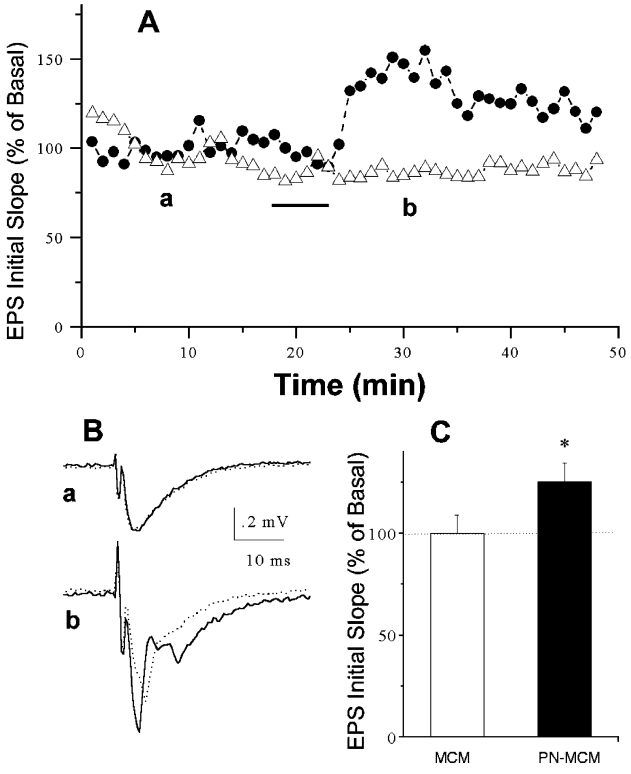


Fig. 7. Neuronal synaptic efficacy following exposure to MCM. Bath application of peripheral nerve stimulated MCM, PN-MCM, (●) (diluted 1:10) significantly enhanced field EPSPs evoked by electric stimulation in hippocampal CA1 region as compared to control MCM (△). The initial slope of the evoked EPSP (measured at peak response) increased to 154.6% of basal level (panel A). Evoked individual EPSPs taken before (dot) and after (solid) bath application of control MCM (upper) and PN MCM (lower) are shown in panel B. Note the increase of EPSP following bath application of PN-MCM. Panel C summarizes synaptic responses to different MCMs. The enhancement in synaptic efficacy following bath application of PN MCM showed a statistical significant difference ($p < 0.05$) when compared to synaptic efficacy assayed after bath application of control MCM. Horizontal bar in panel A indicated the time of bath application of MCM. MCM is MDM-conditioned media.

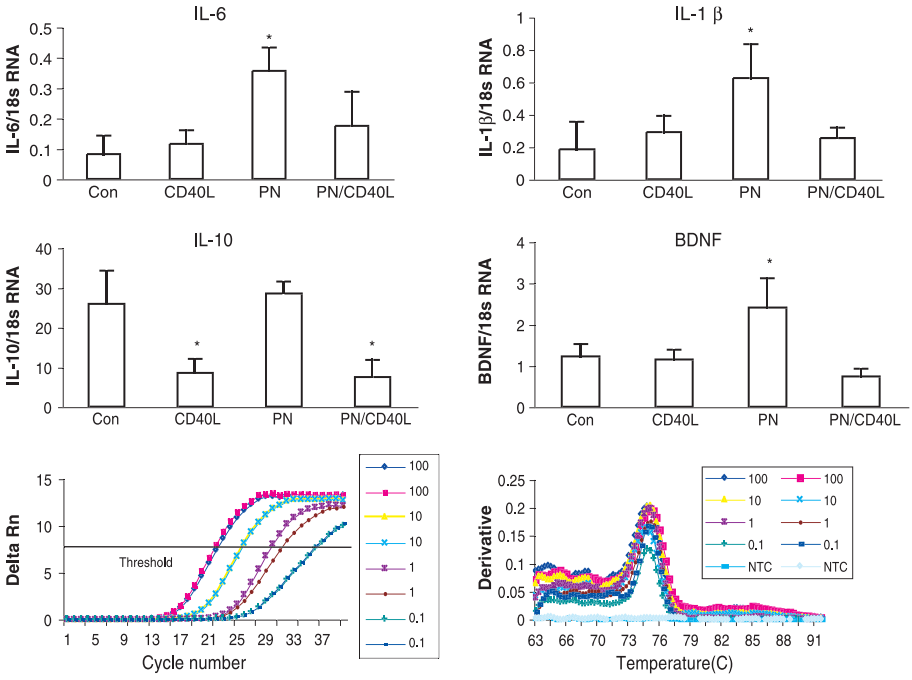


Fig. 8. Real-time quantitative RT-PCR. Normalized expression of neurotrophins and cytokines in MDM treated with CD40L, PN or both are shown as bar graphs where significant increase in BDNF, IL-1β and IL-6 (*$p < 0.05$) was observed in MDM stimulated with PN. A significant reduction in IL-10 expression in MDM treated with CD40L was observed. cDNA obtained from reverse transcription of RNA with random primers was used for the PCR reaction. Dissociation curves were performed for every PCR reaction and showed a single peak demonstrating that no nonspecific products or primer dimers were formed. A representative amplification plot of BDNF using 10-fold dilutions of the cDNA is shown.

control MDM) secrete factors that enhance neuronal survival, differentiation, and synaptic efficacy. To qualitatively determine the putative secretory proteins involved, we analyzed whether stimulated or control MDM differentially express mRNAs of several known neurotrophins and neuroprotective or anti-inflammatory cytokines including BDNF, IL-6, IL-10, and IL-1 β (Kiefer et al., 2001). Real-time RT-PCR of total RNA from MDM stimulated with CD40L or PN or both was performed. Fig. 8 shows that BDNF, IL-1β and IL-6 expressions were observed in macrophages stimulated with PN. In contrast, suppression of IL-10 was observed in macrophages treated with CD40L providing one explanation for the differences in neuronal functional activities between the activated MDM. The DNA melting curve of the product obtained from the dissociation protocol showed a single peak at the Tm of our amplicon demonstrating the absence of primer dimers or nonspecific amplification.

To assess whether increases in mRNA translated into increased protein secretion, ELISA assays were used. Levels of BDNF, and IL-6 along with NGF and NT-3 in MDM-conditioned media were determined and they were found to be consistent in each of the four independent experiments with four separate donors. Table 2 shows that BDNF increased modestly, >3-fold, after CD40L activation of MDM-conditioned media. Stimulation of MDM-conditioned media with PN increased BDNF production >5-fold when compared to control MDM-conditioned media. CD40L plus PN co-stimulation decreased BDNF production dramatically, at levels at or below the level of detection. PN stimulation significantly increased production of NGF above control and CD40L levels (Table 2, $p < 0.05$). Interestingly, PN–MDM-conditioned media was the only activation condition to show a significant increase in NT-3 protein production. The cytokine IL-6 has been shown to have neurotrophic properties alone or in combination with

Table 2
ELISA tests (ng/ml) for PN- and CD40L-stimulated MDM neurotrophins

| MCM neurotrophin | Control | CD40L | PN | CD40L ± PN |
|---|---|---|---|---|
| NGF | 0.115 ± 0.005 | 0.123 ± 0.006 | 0.144 ± 0.004* | 0.125 ± 0.001 |
| NT-3 | 0.042 ± 0.006 | 0.047 ± 0.002 | 0.099 ± 0.008# | 0.048 ± 0.033 |
| BDNF | 4.400 ± 2.500 | 13.95 ± 3.104* | 22.80 ± 3.813# | 0.000 ± 0.000 |
| IL-6 | 1.930 ± 0.024 | 1.973 ± 0.035 | 2.369 ± 0.051* | 2.354 ± 0.130* |

MDM-conditioned media were assayed for the presence of NGF, BDNF, NT-3 and IL-6 using ELISA. Values are means ± S.E.M. of triplicates. Data are representative of four independent experiments (with different donors). Significance as compared to MDM-conditioned media is shown as follows: *Indicates $p < 0.05$, #indicates $p < 0.01$. MDM = monocyte-derived macrophages.

other growth factors. IL-6 levels in PN-, CD40L- or CD40L plus PN–MDM-conditioned media were significantly increased above control MDM-conditioned media ($p < 0.05$, Table 2). Additionally, we assayed tumor necrosis factor alpha (TNF-α). Only CD40L plus PN–MDM-conditioned media showed detectable levels ($9.5 \pm 5.8$ pg/ml). This is likely significant as TNF-α is a putative neurotoxin and may explain why the addition of the two activators showed reduced neurotrophic activities in the MDM-conditioned media.

In attempts to broaden the detection of novel neurotrophins produced by stimulated MDM, ProteinChip® assays (PCT; Ciphergen Biosystems, Palo Alto, CA) were used to analyze MDM-conditioned media. Control MCM showed limited protein peaks within the molecular weights of known neurotrophins (10–20,000 Da, Fig. 9A,B). In contrast, the protein profile of PN–MDM-conditioned media exhibited several major peaks in this range and protein concentrations were dramatically increased (Fig. 9A). One solitary peak at ~ 10,500 Da is currently being characterized. A second major peak corresponded to the size of BDNF. This was confirmed by antibody capture assays (Fig. 9B). Taken together, these data demonstrate that PN stimulation of MDM increased production of neurotrophic factors such as BDNF. Future studies are aimed at further identifying both known and potentially novel proteins secreted by MDM.

## 4. Discussion

The ability of macrophages to act as scavengers, kill microbial pathogens, regulate immune responses, and produce secretory factors is multifaceted, contributing to either neurotoxic or neuroregenerative mechanisms during CNS trauma, metabolic, infectious and degenerative disorders. Previous works using rat models of human disease have demonstrated that injection of PN-activated MDM into areas

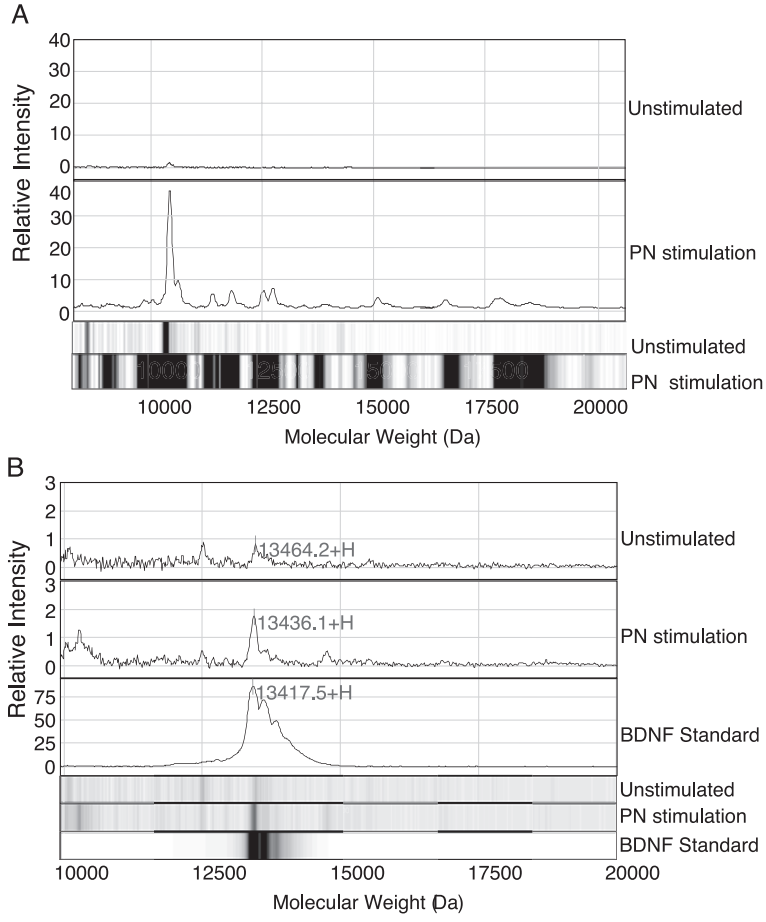


Fig. 9. Surface enhanced laser desorption ionization (SELDI) ProteinChip® analysis of neurotrophins in MCM. General protein profiling of control and PN-MCM is shown in (A). The relative intensity of protein peaks from 10,000 to 20,000 Da is illustrated as a difference map (upper panels) and simulated gel lanes (lower panels). The side-by-side molecular weight profile of unstimulated MCM shows significant increases in protein concentration and number after PN stimulation of MDM. Characterization of protein profiles for BDNF using an antibody capture assay is shown in (B). Recombinant human BDNF protein (R&D Systems) was used as a standard to determine the relative concentration of BDNF in both control and PN stimulated MDM. For PN-stimulated MCM, the peak area of BDNF is 2.7 times greater than control (panel B). These data are normalized to protein mass and intensity by ProteinChip® software. MCM is MDM-conditioned media.

of spinal cord damage promoted neuronal regeneration and recovery of motor function (Franzen et al., 1998; Lazarov-Spiegler et al., 1998; Rapalino et al., 1998; Schwartz et al., 1999b). Similarly, dopaminergic neurons exhibit increased sprouting toward activated microglia and macrophages following neural damage (Batchelor et al., 1999). However, macrophages can lead to increased neuronal damage in a variety of neurodegenerative disorders and following spinal cord injury (Blight, 1992; Minagar et al., 2002; Popovich, 2000; Stoll and Jander, 1999). Activated macrophages have been shown to secrete neurotoxic cytokines (Kiefer et al., 2001), and growth inhibiting proteins (Fitch and Silver, 1997). Taken together, these data suggest that macrophages can respond to a broad range of environmental cues that differentially regulate their neuroprotective and neurotoxic functions.

Our data show that human macrophage neurotrophic activities can be differentially enhanced or reduced following stimulation with distinct environmental cues, human CD40L and/or excised human peripheral nerve. Binding of CD40 ligand to its receptor on macrophages can regulate inflammatory processes (van Kooten, 2000; van Kooten and Banchereau, 1997). It is likely that CD40 ligand is a broad range stimulator of macrophages because of its expression by infiltrating T-cells during autoimmune disorders, endothelial cells lining cerebral microvessels (Mach et al., 1997) and astrocytes (Calingasan et al., 2002). Human peripheral nerve was used to stimulate human MDM because a series of in vivo studies have shown that pre-stimulation of rat MDM with explants of peripheral nerve prior to injection into regions of damaged CNS enhances neuronal regeneration (Schwartz, 2000). A specific role for T lymphocytes has been identified in this system (Hauben et al., 2001) but the importance of macrophages in the regenerative process is not clear. This study presents evidence that human MDM secretory factors are a primary source of neurotrophic factors when stimulated by specific environmental cues.

Treatment of rat cortical neurons with MDM-conditioned media demonstrated increased viability and neurite outgrowth. Both unstimulated and stimulated MDM-conditioned media significantly increased mitochondrial activity in cultured neurons. This experiment provided initial evidence that human MDM secretory factors supported the viability of primary cells of rat cortical neurons. MAP-2 and Tau are microtubule-associated proteins expressed specifically by neurons and their differentiating processes. High molecular weight MAP-2 is localized to neuronal cell bodies, and dendrites while Tau is localized to differentiated axons (Matus, 1991). High molecular weight MAP-2 and Tau ELISA assays were used to quantitate the number of neurons and associated processes expressing these proteins following six days of in vitro differentiation in the presence of MDM-conditioned media. CD40L–MDM-conditioned media, and PN–MDM-conditioned media significantly increased MAP-2 antigen expression compared to neurobasal media supplemented with neurotrophins indicating that both CD40L and PN stimulated MDM secrete factors promoting neuronal survival and dendrite outgrowth. Only rat cortical neurons treated with PN–MDM-conditioned media showed a significant increase in Tau antigen expression indicating that the secretory factors of PN–MDM-conditioned media supported both axonal outgrowth and dendritic outgrowth. CD40L plus PN–MDM-conditioned media showed a significant decrease in MAP-2 and Tau expression compared to CD40L–MDM-conditioned media and PN–MDM-conditioned media (Fig. 1). Neurofilament staining of neuronal cultures following treatment with MDM-conditioned media showed that CD40L–MDM-conditioned media and, to a greater extent, PN–MDM-conditioned media promote neurite outgrowth in these cultures (Fig. 2).

MAP kinase pathway activation in neurons has been demonstrated to be important for neurite outgrowth, regeneration, synaptic plasticity and memory functions in mature neurons (Dougherty et al., 2000; Fukunaga and Miyamoto, 1998; Perron and Bixby, 1999; Sweatt, 2001). Activity of MAPK ERK 1/2 was significantly increased in rat cortical neurons following treatment with MDM-conditioned media, CD40L–MDM-conditioned media, and PN–MDM-conditioned media compared to neurobasal and neurobasal plus NGF and EGF. PN–MDM-conditioned media elicited a significant increase in ERK 1/2 activation compared to other MDM-conditioned media. This ERK 1/2 activation was verified by the ability of the specific MAPK inhibitor, PD98059, to block MAPK pathway activation by MDM-conditioned media. Neuronal ELISA assays for MAP-2 in the presence of PD98059 showed that blocking ERK 1/2 reduced the expression of MAP-2 antigen. While treatment with PD98059 decreased MAP-2 antigen reactivity in cultures, only PN–MDM-conditioned media showed significant increase in antigen expression in our systems. Our data suggest that macrophage secretory products following stimulation with PN activate ERK 1/2 pathways necessary for proper neurite outgrowth. Upregulation of ERK 1/2 kinase activity can enhance gene transcription (Johnson and Lapadat, 2002). Synthesis of proteins such as MAP-2 a,b is necessary for production and maintenance of neuronal processes (Aletta et al., 1988). All MDM-conditioned media conditions increased MAP 2a,b synthesis. Only cortical neurons treated with PN–MDM-conditioned media showed a significant increase in MAP-2 synthesis. CD40L plus PN–MDM-conditioned media showed slight increases in MAP-2 a,b synthesis but this increase was not significantly different from the positive control, neurobasal media plus NGF and EGF or unstimulated MDM-conditioned media. These results show that the secretory components of PN–MDM-conditioned media can specifically enhance the synthesis of proteins necessary for neurite outgrowth and differentiation possibly through the activation of the Erk pathway. Neurotrophins as well as cell adhesion molecules found in the environment of the CNS commonly induce neurite outgrowth, differentiation and synaptic function through acti-

vation of the MAPK pathway (Doherty et al., 2000; Yamada et al., 1997).

Recent studies have shown that activated human MDM release neurotrophic factors (Rapalino et al., 1998; Schwartz, 2000; Schwartz et al., 1999b). It has become clear that neurotrophins promote neuronal survival and differentiation and play essential roles in synaptic transmission and plasticity (Schinder and Poo, 2000; Thoenen, 1995). To determine the influence of PN–MDM secretory products on synaptic function, we examined the effects of PN–MDM-conditioned media on evoked EPSPs in rat hippocampal slices in vitro. These results demonstrated that only PN–MDM-conditioned media significantly enhanced neuronal EPSPs. Notably, these effects were recorded at the level of the synapse (within Schaffer collaterals commissural to CA1 pyramidal cells), supporting the biological significance of these test results. Most striking, the PN–MDM-conditioned media-induced enhancement was long lasting (see Fig. 5A). The mechanism(s) underlying the enhancement of synaptic transmission following treatment with PN–MDM-conditioned media is not clear. It may represent the effects of some neurotrophins (for example, BDNF) released by PN-stimulated MDM. Since BDNF plays a role in the regulation of synaptic strength (Yamada et al., 2002) and significant higher concentration of BDNF was detected in PN–MDM-conditioned media by SELDI ProteinChip analysis, we postulate that BDNF may be one of the crucial factors causing the enhancement of hippocampal synaptic transmission. These results indicate that the PN–MDM-conditioned media could amplify long-term potentiation (LTP) by enhancing synaptic transmission in the CA1 region of the hippocampus. This effect of PN–MDM-conditioned media on synaptic transmission may reflect part of its neuroprotective nature as observed by other investigators (Schinder and Poo, 2000; Thoenen, 1995).

Deciphering the specific neurotrophic factors secreted by peripheral blood macrophages and the mechanisms leading to their production could have implications for treatment of CNS disorders. While the levels of the growth factors produced by these MDM varied between monocyte donors, consistent neurotrophic activity of the MDM-conditioned media was still observed. Real-time PCR and ELISA analysis of the secretory products of unstimulated MDM and stimulated MDM identified that specific neurotrophin mRNA and protein products were increased in MDM-conditioned media. Cytokines IL-1β, IL-1 0, and IL-6 were significantly increased in conditioned media of PN-stimulated MDM. These cytokines have diverse action in the brain and may have either neurotoxic or neurotrophic actions in the CNS. Similar to the results presented here, IL-6 and IL-1β at specific concentrations promote the survival of multiple neuronal cell types (Edoff and Jerregard, 2002; Marz et al., 1998; Thorns et al., 2002). Specifically, IL-6 has been recently shown to activate STAT signaling necessary for differentiation of PC12 cells and may have an important role not only in increased survival of neurons but also regulation of the neurite outgrowth necessary for the development of synaptic function and regeneration following injury (Wu and Bradshaw, 2000).

Our results show that human MDM, under normal homeostatic conditions possess neurotrophic properties. These neurotrophic properties can be increased following exposure to specific stimulation such as CD40L, and in particular, excised human PN. CD40–CD40L ligation on macrophages is normally studied in a background of particular disease states such as lentiviral infection (Bragg et al., 2002; Cotter et al., 2001) and Alzheimer's disease (Calingasan et al., 2002; Tan et al., 1999) and is associated with an increase in the synthesis of pro-inflammatory cytokines. Nonetheless, our studies demonstrate an alternative role for CD40–CD40L interactions. Recently, cortical neurons have been shown to express CD40 and CD40–CD40L interactions support neuronal development, survival and protection from apoptosis (Tan et al., 2002). Stimulation of macrophages with PN exhibited the highest neurotrophic properties in all cellular assays tested in this report. An increase in synthesis of a battery of neurotrophins (as well as a decrease in synthesis of neurotoxins such as TNF-α) is likely to be an important component of the increased ability of PN–MDM-conditioned media to support neuronal survival, differentiation, and synaptic activity. In our studies, the effects of conditioned media from CD40L- or PN-stimulated MDM were most likely due to an increase in neurotrophic secretory products of macrophages and not CD40L or PN directly stimulated MDM were washed three times prior to conditioning neurobasal media used for culturing cortical neurons. Neurotrophic properties can be decreased by co-stimulation with CD40L and excised human PN. Co-stimulation of macrophages with CD40L plus PN may be similar to the microenvironment of Alzheimer's disease (Town et al., 2001) or HIV (D'Aversa et al., 2002), where CD40–CD40L interaction on immune cells is associated with increased neurotoxicity. Many of the neurotoxic effects of CD40L plus PN–MDM-conditioned media may be due to secondary activation of astrocytes since CD40L plus PN–MDM-conditioned media could variably promote the survival and differentiation of PC12 cells in an astrocyte-free culture system. To address the underlying cellular mechanisms controlling the switch from neurotrophic to neurotoxic MDM-conditioned media, our ongoing studies are directed at investigating the regulation of intracellular signaling pathways and gene expression in MDM that affect one outcome over the other.

Neurotrophins such as NGF, NT-3, BDNF, shown here to be secreted by MDM, are associated with increased survival, differentiation and synaptic function of CNS neurons Together, these neurotrophin molecules may work to activate ERK function in neurons and promote both survival and neurite outgrowth (Patapoutian and Reichardt, 2001). PCT analysis provided highly sensitive comparisons between MCM samples. Ciphergen PCT was used since it profiles the molecular mass of proteins that differ by less

than 0.2% (200 ppm) and permits femtomole detection. Consequently, PCT can be used to accurately calculate the mass of compounds ranging from 1000 Da to 500 kDa (Austen et al., 2000; Davies et al., 1999; Merchant and Weinberger, 2000; Vlahou et al., 2001). PCT analysis showed that BDNF levels were higher in PN-stimulated than in unstimulated MDM-conditioned media and may represent one pathway for neuronal trophic activities by MDM. Indeed, BDNF supports the survival and differentiation of CNS neurons by activation of MAP kinases (Cavanaugh et al., 2001; Han and Holtzman, 2000), PI3K (Dolcet et al., 1999) or a by co-activation of both pathways (Hetman et al., 1999; Kelly-Spratt et al., 2002). BDNF increases the MAP-2 synthesis required for neurite outgrowth (Drubin and Hirokawa, 1998; Greene et al., 1983). Upregulation of BDNF and other neurotrophins by macrophages following spinal cord injury in rats may help restore neuronal function or aid in repair of damaged connections (Batchelor et al., 2002b; Ikeda et al., 2001). Moreover, this trophic factor can enhance long-term potentiation as stated above. Interestingly, recent works showed that enhancement of long-term potentiation by BDNF required ERK activation coupled to CREB and increased Arc synthesis (Ying et al., 2002). While BDNF may account for many of the MDM neurotrophic properties observed in these studies, further characterization of the role of this particular neurotrophin is ongoing. It is more likely that the balance of all MDM secretory products, both cytokines and neurotrophins, controls their neurotrophic (or neurotoxic) function in the CNS.

Taken together, these data support the idea that macrophage function can be modulated by the specific stimulation encountered in their environment. Further investigation into macrophage microenvironments favoring the production of known and novel neurotrophic factors will provide valuable insight into the potential therapeutic properties of MDM to limit damage to and promote regeneration of the central nervous system following injury or during neurodegenerative diseases.

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