

Fibroblast Growth Factor Modulates HIV Coreceptor CXCR4 Expression by Neural Cells

Virginia J. Sanders,¹ Ian P. Everall,³ Robert W. Johnson,¹ Eliezer Masliah,^{2*} and HNRC Group

¹Department of Neurosciences, University of California, San Diego, La Jolla, California

²Departments of Neurosciences and Pathology, University of California, San Diego, La Jolla, California

³Departments of Neuropathology and Psychological Medicine, Institute of Psychiatry, DeCrespigny Park, London, United Kingdom

Recent studies suggest that the chemokine receptor CXCR4 may be involved in mediating the neurodegenerative process in the brains of patients with acquired immunodeficiency disease (AIDS). In this context, we hypothesize that neurotrophic factors, such as fibroblast growth factor (FGF), might protect against human immunodeficiency virus (HIV)-mediated neurotoxicity via regulating the expression of CXCR4 in neural cells. For this purpose, levels of CXCR4 were determined in neuronal and glial cell lines after FGF1 and 2 treatment. In addition, levels of CXCR4 immunoreactivity were associated with levels of FGF1 immunoreactivity in the brains of HIV-positive patients. These studies showed that neuronal CXCR4 levels decreased in a dose-dependent manner after exposure to FGF. Conversely, glial CXCR4 was increased in a dose-dependent manner after FGF2 treatment. These effects were dependent on the FGF receptor tyrosine kinase signaling pathway, because FGF-induced effects on CXCR4 were blocked by the tyrosine kinase inhibitor, 5'-deoxy-5'-methylthioadenosine, or by anti-FGF receptor antibody. Stromal cell-derived factor-1, the ligand for CXCR4, and HIV gp120 neurotoxicity was attenuated by FGF1 in a dose-dependent manner *in vitro*, further supporting physiological relevance. In the brains of AIDS patients, the levels of neural CXCR4 immunoreactivity were inversely associated with FGF levels. Taken together, these results support the possibility that the neuroactive effects of FGF in HIV encephalitis might be mediated through regulation of the expression of CXCR4. *J. Neurosci. Res.* 59:671–679, 2000. © 2000 Wiley-Liss, Inc.

Key words: neuron; astroglia; neurodegeneration; HIV encephalitis

Human immunodeficiency virus (HIV)-1 infection of the central nervous system (CNS) is frequently observed in acquired immunodeficiency disease (AIDS) patients. However, there is often a discrepancy among the presence of virus, degree of neuronal damage, and behavioral abnormalities (i.e., dementia). Neurodegeneration may be

present without high CNS viral loads, or, conversely, virus and/or HIV encephalitis may be seen in the CNS with little or no neuronal damage. The broad spectrum of behavioral and neuropathological abnormalities may reflect a sensitive balance between neuroprotective and neurotoxic factors in the CNS of HIV-infected patients.

Recent studies have suggested that fibroblast growth factor (FGF) might protect against neurotoxic factors in HIV. FGF1 and FGF2 (acidic and basic, respectively) are pluripotent growth factors that have been localized in the CNS and play a variety of roles in normal homeostasis and injury repair. FGF1 is localized to neurons in the temporal cortex, the thalamus, the substantia nigra cortex, and basal ganglia, while FGF2 is produced by astrocytes and subpopulations of neurons, i.e., CA2 pyramidal neurons (Eckenstein et al., 1994). Receptors for FGF1 and 2 are localized to neurons, astrocytes, and microglia (Balaci et al., 1994). Given the distribution of FGF and receptors, it most likely acts in both an autocrine and paracrine manner. These growth factors enhance neuronal survival and

The San Diego HIV Neurobehavioral Research Center (HNRC) group is affiliated with the University of California, San Diego, the Naval Hospital, San Diego, and the San Diego VA Medical Center, and includes: Igor Grant, M.D., Director; J. Hampton Atkinson, M.D., Codirector; Thomas D. Marcotte, Ph.D., Center Manager; James L. Chandler, M.D., and Mark R. Wallace, M.D., Coinvestigators, Naval Hospital San Diego; J. Allen McCutchan, M.D., P.I. Neuromedical Component; Stephen A. Spector, M.D., P.I. Virology Component; Robert K. Heaton, Ph.D., P.I. Neurobehavioral Component; Terry Jernigan, Ph.D., and John Hesselink, M.D., Co-P.I.s Imaging Component; Eliezer Masliah, M.D., P.I. Neuropathology Component; J. Allen McCutchan, M.D., J. Hampton Atkinson, M.D., and Ronald J. Ellis, M.D., Ph.D., Clinical Trials Component; Daniel R. Masys, M.D., P.I. Data Management Component; and Ian Abramson, Ph.D., P.I. Statistics Unit.

Contract grant sponsor: National Institutes of Health; Contract grant numbers: MH12549-01, MH45294, MH59745, DA12065.

*Correspondence to: Dr. Eliezer Masliah, Departments of Neuroscience and Pathology, 9500 Gilman Drive, University of California, San Diego, La Jolla, CA 92093-0624. E-mail: emasliah@ucsd.edu

Received 15 September 1999; Accepted 3 October 1999

can attenuate neurotoxicity in vitro (Himmelseher et al., 1996, 1997; Mark et al., 1997) and in vivo (Maggio et al., 1997; Wirth et al., 1996; Yang and Cui, 1998). While the mechanism(s) by which FGF is neuroprotective have not been fully elucidated, it has been demonstrated that FGF binds to a tyrosine kinase receptor and thus may activate a number of intracellular signaling pathways. FGF has also been shown to be translocated to the nucleus and exert its effects there.

One possible modulator of neurodegeneration is the chemokine receptor, CXCR4. In the periphery, it is involved in the directional migration of immune cells in response to its ligand, stromal cell-derived factor-1 (SDF-1). It also acts as coreceptor, along with CD4, in binding and fusion of HIV-1, specifically interacting with the viral envelope protein gp120. In the CNS, CXCR4 has been localized to microglia, astrocytes, and neurons (Lavi et al., 1997; Sanders et al., 1998). The function of the receptor on neurons is unknown, although knock-out studies suggest a role in neuronal migration during development (Zou et al., 1998). The significance of CXCR4 as a coreceptor for HIV infection of neural cells is under investigation, since infection of neurons and astrocytes is limited or absent. However, gp120 may bind to CXCR4 and elicit signaling without subsequent infection (Davis et al., 1997). Furthermore, gp120 and SDF-1 have been shown to result in neuronal apoptosis via CXCR4 in vitro (Hesseltger et al., 1998; Meucci et al., 1998; Ohagen et al., 1999). Thus the chemokine receptor CXCR4 may play a role in neuronal damage seen in HIV encephalitis (HIVE).

Modulation of CXCR4 expression on neurons may be a mechanism for neuroprotection or neurodegeneration. FGF2 has been shown to upregulate expression of CXCR4 on endothelial cells in vitro (Feil and Augustin, 1998), contributing to its angiogenic capabilities. We propose that a similar mechanism is involved in the neuroprotective actions of FGF in the CNS. The purpose of this study is to determine whether FGF modulates neuronal and glial CXCR4 expression in vitro and whether FGF is associated with CXCR4 levels in the CNS of patients with HIV.

MATERIALS AND METHODS

Human Tissue

Postmortem human brain tissues from 25 HIV-seropositive patients were obtained in accordance with UCSD human IRB approval. Brain tissues from five non-HIV patients were included for examination of qualitative differences. Midfrontal and basal ganglia tissues were examined either as 8- μ m paraffin sections or as 40- μ m vibratome sections stored in glycerine cryoprotectant media at -20°C , depending on qualitative or quantitative analysis, respectively.

Immunocytochemical/Immunofluorescent Microscopy

Monoclonal antibody to human fibroblast growth factor-1 was obtained from Sigma (St. Louis, MO) and used at 2.5 $\mu\text{g}/\text{ml}$. Monoclonal anti-human fibroblast growth factor-2 was

obtained from Calbiochem (San Diego, CA) and used at 0.5 $\mu\text{g}/\text{ml}$. CXCR4 was localized using monoclonal antibody to CXCR4/fusin from Pharmingen (La Jolla, CA) at a 1 $\mu\text{g}/\text{ml}$.

Immunocytochemical staining was performed using the avidin-biotin complex (ABC) method according to instructions in the Vector Elite kit (Burlingame, CA). Paraffin sections were cleared in Hemo-De (Fisher Scientific, Rockville, MD), rehydrated, and antigen retrieval was performed using Dako Target Retrieval Solution (Carpenteria, CA) for 30 minutes, 95°C . All tissues were incubated with 3% hydrogen peroxide for 15 minutes to block endogenous peroxidase, and blocked in 3% normal horse or goat serum. Sections were incubated in primary antibody diluted in 2% horse or goat serum overnight at 4°C . Sections were rinsed in phosphate-buffered saline (PBS) and incubated with a 1:200 dilution of either biotinylated horse anti-mouse antibody or goat anti-rabbit antibody for 2 hours room temperature. After rinsing, sections were treated with ABC solution for 1 hour. Color reaction was developed by treating with 0.01% 3,3'-diaminobenzidine and 0.0015% H_2O_2 . Negative controls were performed by omitting the primary antibody and incubating with diluent only. Fluorescent detection was performed as described above except that fluorescein-labeled or rhodamine-labeled secondary antibodies were used at a 1:100 dilution.

Quantitation

Semiquantitative measurement of CXCR4 immunoreactivity in neurons in optical density (OD) was performed using a Leica Quantimet 570c image analysis system. Pyramidal neurons from layer 5 in the midfrontal cortex and aspiny striatal neurons from the basal ganglia were measured (10 cells in each region) and averaged as previously described (Everall et al., 1997). Similar analysis of fluorescent imaging of FGF1 was performed with laser confocal scanning microscope (MRC 1024, Biorad, Watford, UK) as previously described (Masliah et al., 1992), and staining levels were assessed by evaluating pixel values within labeled cells using Biorad LaserSharp software.

Cell Culture

C6 glioblastoma cells (ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. For protein quantitation, cells were grown in 75- cm^2 flasks for 5 days after passaging and then treated with growth factors. Cells were removed from flasks by incubating with approximately 2 ml of 0.25% trypsin for 4 minutes. For imaging, cells were plated onto glass coverslips coated with poly-L-lysine (200 $\mu\text{g}/\text{ml}$).

SH-SY5Y cells, a noradrenergic subclone of the neuroblastoma cell line, SK-N-SY, were grown in DMEM supplemented with 10% FBS, 1% L-glutamine, and 0.1% gentamicin. Cells were replated at 2×10^5 cells/ cm^2 in this media. After 24 hours, media used to differentiate the cells was composed of DMEM, $1 \times \text{N}2$ supplements (Gibco, Gaithersburg, MD) and 10^{-5} M all-trans retinoic acid. Media with retinoic acid was replaced every 2 days. Cells were treated on day 7 postreplate.

Growth factors used were recombinant human FGF1 (Sigma), FGF2 (Calbiochem, La Jolla, CA), nerve growth factor (NGF), 7S (Sigma), brain-derived neurotrophic factor (BDNF) (Genzyme, Cambridge, MA), or leukemia inhibitory factor (LIF)

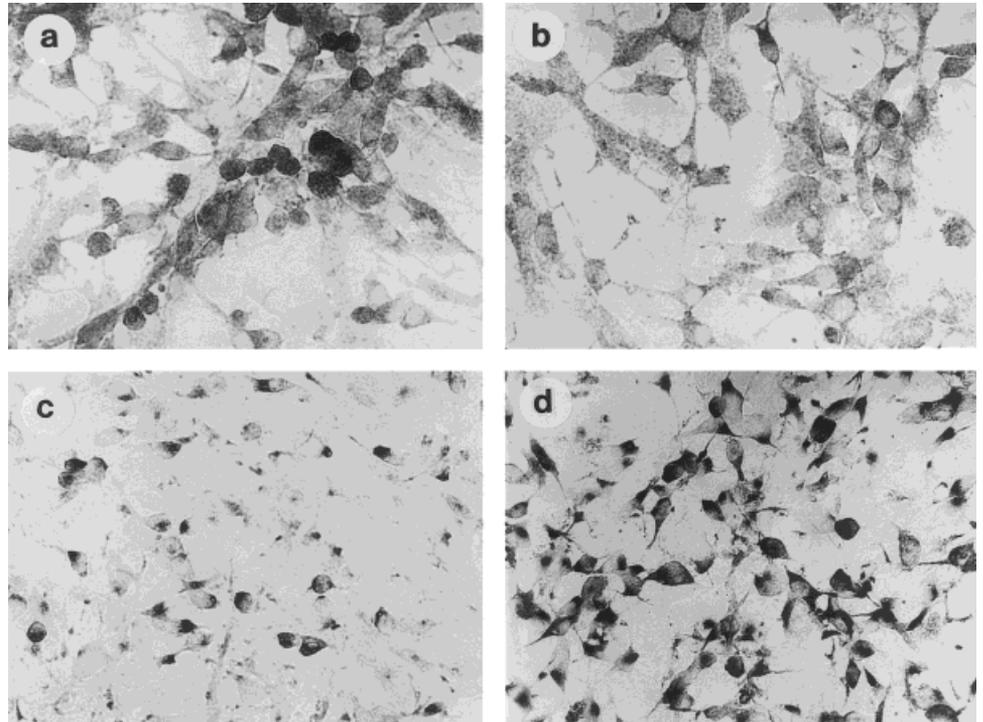


Fig. 1. Modulation of immunoreactivity for CXCR4 by fibroblast growth factor (FGF). **a:** CXCR4 is expressed by SH-SY5Y cells (day 7 postreplate) grown on poly-L-lysine-coated coverslips. **b:** Staining intensity is decreased after 4 hours of treatment with 10 ng/ml FGF1. **c:** CXCR4 is expressed by C6 astroglia cells (day 5 postreplate). **d:** Staining intensity is increased after 4 hours of treatment with 5 ng/ml FGF2. (200 \times magnification).

(R&D Systems, Minneapolis, MN). Blocking experiments were performed using the tyrosine kinase inhibitor, 5'-deoxy-5'-methylthioadenosine (MTA) (Sigma), and/or antibodies against FGF receptors 1 and 2 (Santa Cruz Antibodies, Santa Cruz, CA).

Western Blot

Protein analysis was performed on cell culture homogenates. Cells were removed by mild trypsinization (C6) or scraping (SH-SY5Y) and homogenized in 300 μ l of homogenization buffer (1.0 mM HEPES, 5.0 mM benzamidine, 2.0 mM β -mercaptoethanol, 3.0 mM EDTA, 0.5 mM magnesium sulfate, 0.05% sodium azide, and 0.01 mg/ml protease inhibitor, leupeptin). Protein (1 μ g/ μ l) was electrophoresed on a 12% acrylamide Tris-Glycine gel and transferred to nitrocellulose membrane (0.45 μ m). Nonspecific binding was blocked with 1 hour preincubation in 0.1% Tween 20/PBS, followed by incubation overnight at 4 $^{\circ}$ C with 1 μ g/ml primary antibody, rabbit anti-human CXCR4 (Calbiochem) in 3% bovine serum albumin (BSA)/PBS. After rinsing in 0.1% Tween/PBS, the membrane was incubated for 2 hours at 4 $^{\circ}$ C with 125 I Protein A which binds to rabbit IgG. Immunoreactivity was visualized and quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

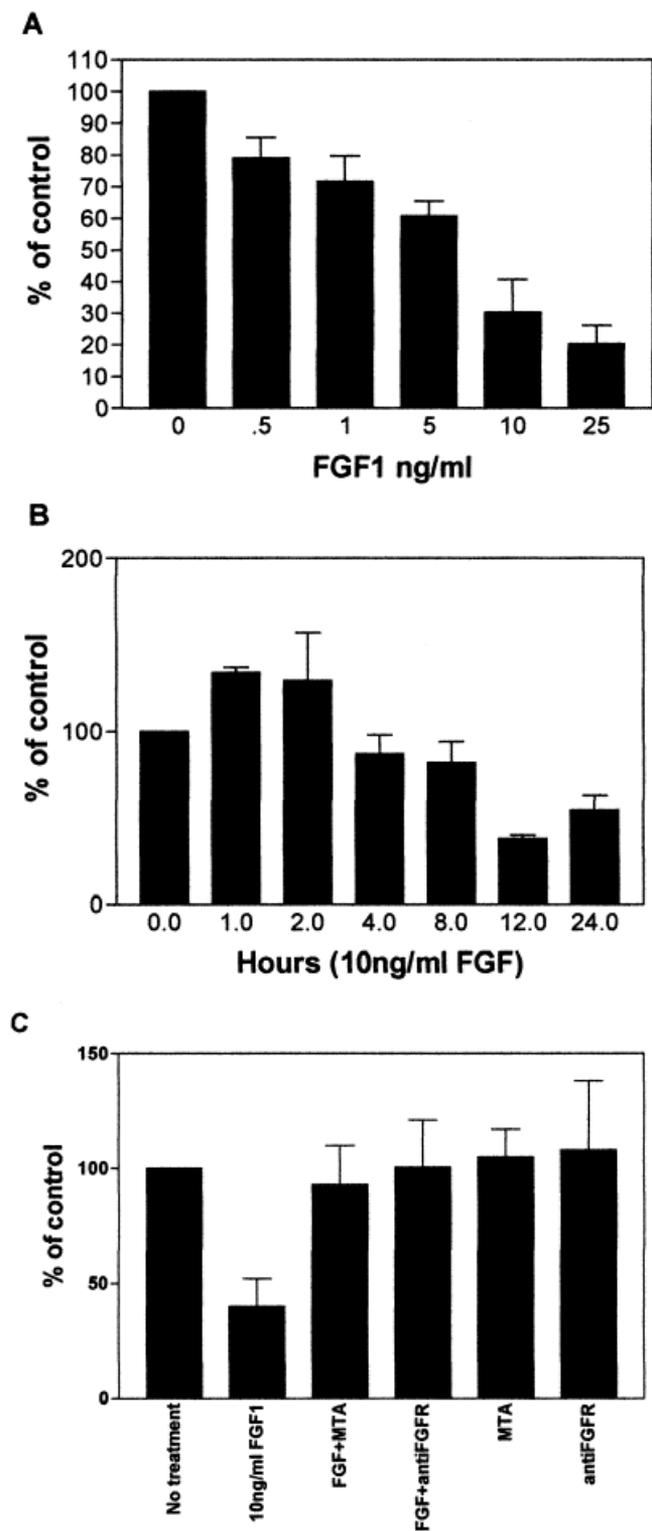
Cell Proliferation Assay

To determine whether FGF induced significant proliferation of C6 glial cells, MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium]-based cell proliferation assay (Promega CellTiter 96 Aqueous Proliferation Assay) was performed per instructions. Briefly, C6 cells were plated in 96-well plates. At day 5, cells were treated

with 5 ng/ml FGF2 for 1, 2, 4, 8, and 12 hours; samples were run in quadruplicate. Twenty microliters of reaction mixture of the tetrazolium compound, MTS, and an electron coupling reagent, phenazine methosulfate (PMS), was added to each sample. After 2 hours incubation at 37 $^{\circ}$ C in a humidified 5% CO₂ atmosphere absorbance at 490 nm was recorded using an enzyme-linked immunosorbent assay (ELISA) plate reader.

Lactate Dehydrogenase Release Assay

Previous studies have shown that neuronal exposure to gp120 and SDF1 results in apoptosis via CXCR4 (Hesselgesser et al., 1998; Meucci et al., 1998; Ohagen et al., 1999). To determine whether the neuroprotective effects of FGF were physiologically relevant by ameliorating SDF1 or HIV gp120 toxicity in neurons, SH-SY5Y exposed to the CXCR4 ligand, SDF1 α , or to HIV gp120 were analyzed with the CytoTox96 $\text{\textcircled{R}}$ Assay (Promega Corp., Madison, WI) which measures lactate dehydrogenase (LDH) release (cytotoxicity). Briefly, SH-SY5Y cells were plated as described above in 96-well plates. On day 5 postreplate, cells were pretreated with 0, 1, 5, 10 ng/ml FGF1 for 12 hours to reduce CXCR4 levels; they were then exposed to either 25 nM SDF1 α (Intergen Co. Purchase, NY) or 400 pM gp120IIIIB (Intracell Corp. Issaquah, WA) for 48 hours. All experimental groups were run in triplicate. As per assay instructions, half of the wells were treated with 0.8% Triton-X solution for 45 minutes to determine maximal LDH release; 50 μ l of media from each well was transferred to another 96-well plate; addition of 50 μ l/well substrate buffer resulted in the enzymatic formation of a red formazan product which was quantitated using a standard ELISA reader at 492 nm. The final amount of color produced is proportional to the number of cells lysed.



Results were determined as percentage of cell survival $(1 - [\text{experimental absorbance}/\text{maximal lysis absorbance}] \times 100\%)$ and reported as percent change compared to untreated control SH-SY5Y cells.

RESULTS

FGF1 Decreases CXCR4 in Neuronal Cells

As there was no access to fetal material to establish primary human neurons, we used the human neuroblastoma cell line SH-SY5Y, differentiated into postmitotic cells with retinoic acid. This cell line has been shown previously to express CXCR4 (Loetscher et al., 1994). Consistent with these results, CXCR4 was detected by immunocytochemistry (Fig. 1a,b) and by Western blot.

To determine the effects of FGF on CXCR4 receptor expression, SH-SY5Y cells were exposed to increasing concentrations of FGF1 for 4 hours. Neuronal CXCR4 expression was decreased in a dose-dependent manner by FGF1. At 0.5 ng/ml FGF1, level of expression was 76% of control (untreated SH-SY5Y cells) and decreased to 15.5% of control after treatment with 25 ng/ml FGF1 (Fig. 2A).

To determine the time course of the observed decrease in protein concentration, SH-SY5Y cells were treated with 10 ng/ml of FGF1 for 1, 2, 4, 8, 12, and 24 hours (Fig. 2B). There was an initial increase in protein levels seen at 1 and 2 hours after addition of FGF1 (134% and 129%, respectively). After 4 hours of treatment, protein level was 87% of untreated controls and decreased to maximal reduction of 38% of control at 12 hours. After 24 hours of treatment, CXCR4 levels were 54.5% on untreated controls.

To establish that the observed effect was through FGF receptor signaling directly, we blocked FGF receptor signaling by inhibiting its tyrosine kinase activity with 5'-methylthioadenosine (MTA), which has been shown to specifically inhibit FGF activity (Maher, 1993), or by blocking binding of FGF with excess concentrations of antibodies to FGF receptors 1 and 2. Cells were untreated (control), or treated with one of the following: 10 ng/ml FGF1, 10 ng/ml FGF plus 10 mM MTA, 10 ng/ml FGF plus 5 $\mu\text{g}/\text{ml}$ anti-FGF receptor 1 and 2 antibodies, MTA alone, or anti-receptor antibodies alone (Fig. 2C). MTA was added to the cell culture media 30 minutes prior to FGF exposure, since pretreatment has been shown to result in greatest reduction of FGF receptor tyrosine kinase activity. Treatment of SH-SY5Y cells with 10 ng/ml FGF1 reduced protein levels to 40% of untreated control. Pretreatment with MTA or treatment with FGF receptor

Fig. 2. CXCR4 protein levels are decreased by FGF in SH-SY5Y cells. Semiquantitation of SH-SY5Y cell lysate protein levels by Western blot. Values are given as percentage of untreated controls. **A:** CXCR4 expression is decreased in a dose-dependent manner. **B:** CXCR4 expression decreases over time. **C:** FGF1-induced decrease of CXCR4 is inhibited by treatment with 5'-methylthioadenosine (MTA), an FGF receptor tyrosine kinase inhibitor. Error bars represent standard error.

blocking antibodies inhibited this reduction (93% and 100.5% of control, respectively). Treatment with MTA or blocking antibodies alone did not significantly alter CXCR4 protein levels.

To establish that the observed effects were specific to FGF, SH-SY5Y cells and C6 cells were treated with NGF, BDNF, or LIF (all 10 ng/ml) for 8 hours (SH cells) or 1 hour (C6). These trophic factors did not induce any changes in receptor protein levels compared to untreated controls as determined by Western blot analysis (data not shown).

FGF Increases CXCR4 in Astroglial Cells

Since FGF exerts mitogenic effects on astroglia, contributing to astrogliosis, we hypothesized that FGF would increase CXCR4 levels in a manner similar to its effects on endothelial cells. C6 glioma cells were treated with increasing concentrations of FGF2 for 4 hours. Astroglial CXCR4 expression was increased in a dose-dependent manner by FGF (Fig. 3A). Treatment with FGF2 resulted a maximal increase in receptor expression at 5 ng/ml (189% above untreated control). Higher concentrations of FGF (10 and 25 ng/ml) elicited a smaller increase. Increase in immunoreactivity for CXCR4 was also observed in C6 cells (Fig. 1c,d).

The time course of FGF’s increasing CXCR4 protein level was examined. Initial experiments similar to those for neuronal cells (up to 24 hours) demonstrated that, after treatment with 5 ng/ml, expression increased very rapidly and plateaued as early as 1 hour (data not shown). Therefore, we examined protein levels at 5, 15, 30, 45, and 60 minutes after treatment with 5 ng/ml FGF. Expression was up to 139% of untreated control by 5 minutes of treatment. Levels increased steadily, reaching 188% of control at 45 minutes and 196% of control at 1 hour (Fig. 3B).

To test the specificity of FGF-induced increase, FGF receptor tyrosine kinase activity was inhibited by pretreatment with MTA, or FGF receptor binding was inhibited by antibodies to the receptors for FGF. C6 cells were untreated (control), or treated with one of the following: 5 ng/ml FGF2, 5 ng/ml FGF plus 10 mM MTA, 5 ng/ml FGF plus 5 μg/ml anti-FGF receptor 1 and 2 antibodies, MTA alone, or anti-receptor antibodies alone (Fig. 3C). Increase in CXCR4 protein levels in response to FGF treatment (239% of control) was blocked by 30-minute pretreatment with 10 mM MTA. The increase was also inhibited by coincubation with anti-FGFR1 and FGFR2 antibodies. MTA or antibody treatment alone did not-

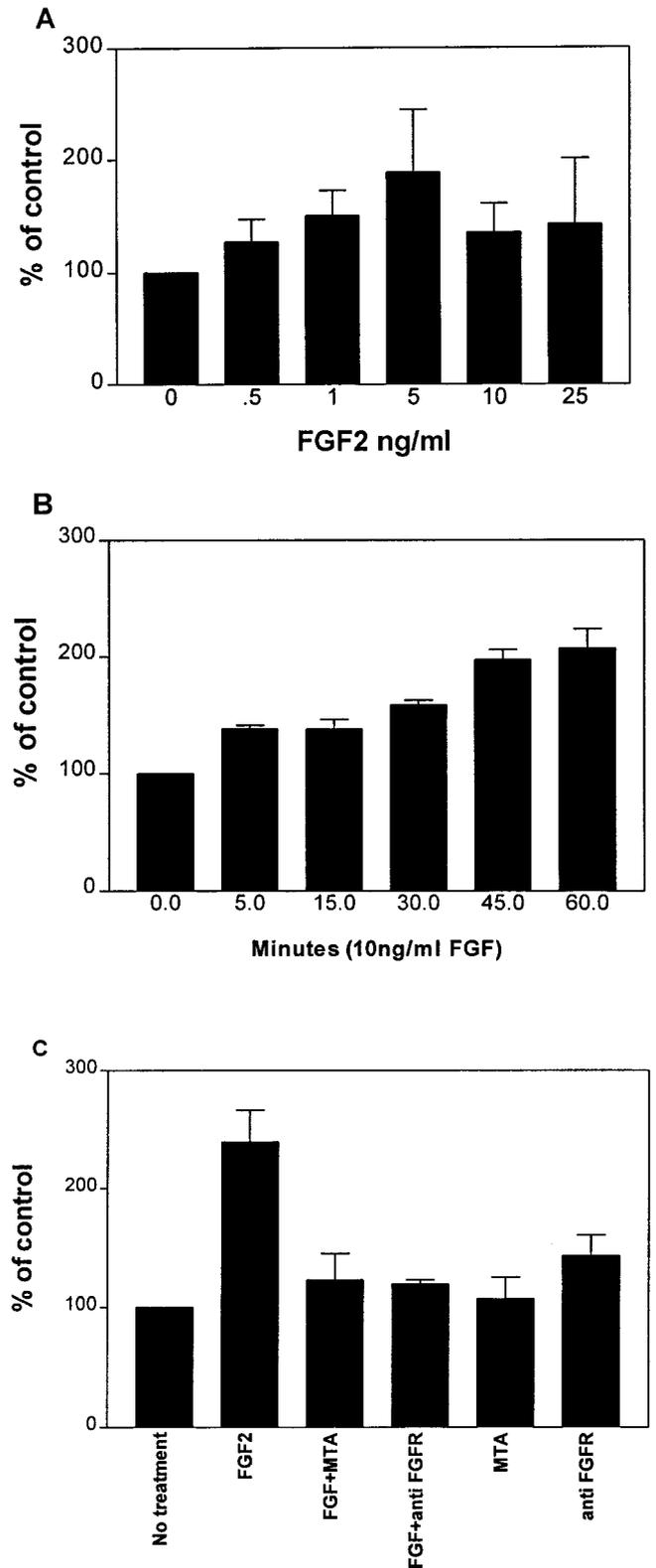


Fig. 3. CXCR4 protein levels are increased by FGF in C6 astroglial cells. Semiquantitation of cell lysate protein levels by Western blot. **A:** CXCR4 expression is increased in a dose-dependent manner. **B:** CXCR4 expression increases over time. **C:** FGF1-induced increase in CXCR4 is inhibited by treatment with MTA, FGF receptor tyrosine kinase inhibitor. Error bars represent standard error.

significantly alter receptor expression compared to untreated control.

To determine whether the increase in CXCR4 receptor protein levels was related to the mitogenic response of astrocytes to FGF, an MTS-based proliferation assay was performed on C6 cells at various time points after incubation with 10 ng/ml FGF2 (data not shown). There was no significant difference between cell number (viability) of untreated control astrocytes and those treated for 4 hours (the time point for the dose response assay).

FGF Protects Against SDF1- or HIV gp120-Induced Neurotoxicity in a Dose-Dependent Manner

To further test that the neuroprotective effects of FGF are physiologically relevant and are protective by modulating CXCR4 levels, we measured cell survival of SH-SY5Y pretreated for 12 hours with various doses of FGF1 and then exposed to SDF1 α or HIV gp120IIIB for 48 hours. Cells were pretreated for 12 hours with FGF to ensure maximal reduction of CXCR4 levels. SDF1 α alone induced a 25.6% decrease in SH-SY5Y cell survival compared to untreated SH-SY5Y cells (Fig. 4A). Pretreatment of neuronal cells with 1 ng/ml FGF1 ameliorated SDF1 α toxicity, resulting in only a 13% decrease in survival compared to untreated controls. Higher concentrations of FGF1 (5 and 10 ng/ml) increased cell survival to 1.3% and 7.5% increase above control, respectively (analysis of variance [ANOVA] $F(4,13) = 4.259$; $P = 0.02$). Exposure of neuronal cells to HIV envelope protein gp120IIIB resulted in a 23% decrease in cell survival compared to untreated control (Fig. 4B). Pretreatment of neuronal cells with 1 ng/ml FGF1 reduced HIV gp120 neurotoxicity, resulting in only a 9% decrease in survival compared to untreated controls. Cell survival was 3.8% and 8.6% above control after pretreatment with 5 and 10 ng/ml FGF1, respectively (ANOVA $F(4,12) = 3.561$, $P = 0.039$). Taken together, FGF1 ameliorated the toxic effect of SDF1 α or gp120IIIB in a dose-dependent manner.

Neuronal FGF1 Immunoreactivity is Associated With Decreased Neuronal CXCR4 Immunoreactivity in the Brains of AIDS Patients

To provide in vivo disease relevance to our in vitro observations, we examined the expression of FGF1 and CXCR4 in postmortem tissue from HIV-infected patients with varying degrees of CNS neurodegeneration. Consistent with previous results (Eckenstein et al., 1994), FGF1 immunostaining was localized to neuronal soma in both midfrontal and basal ganglia. FGF2 was seen in neuronal and astrocytic nuclei with occasional weak perinuclear and cytoplasmic staining in astrocytes. Staining for CXCR4 was present on neurons, astrocytes, and endothelial cells. Qualitative examination between HIV-infected and non-infected control tissue revealed a greater number of CXCR4-positive astrocytes, particularly in the white matter (Fig. 5A–D).

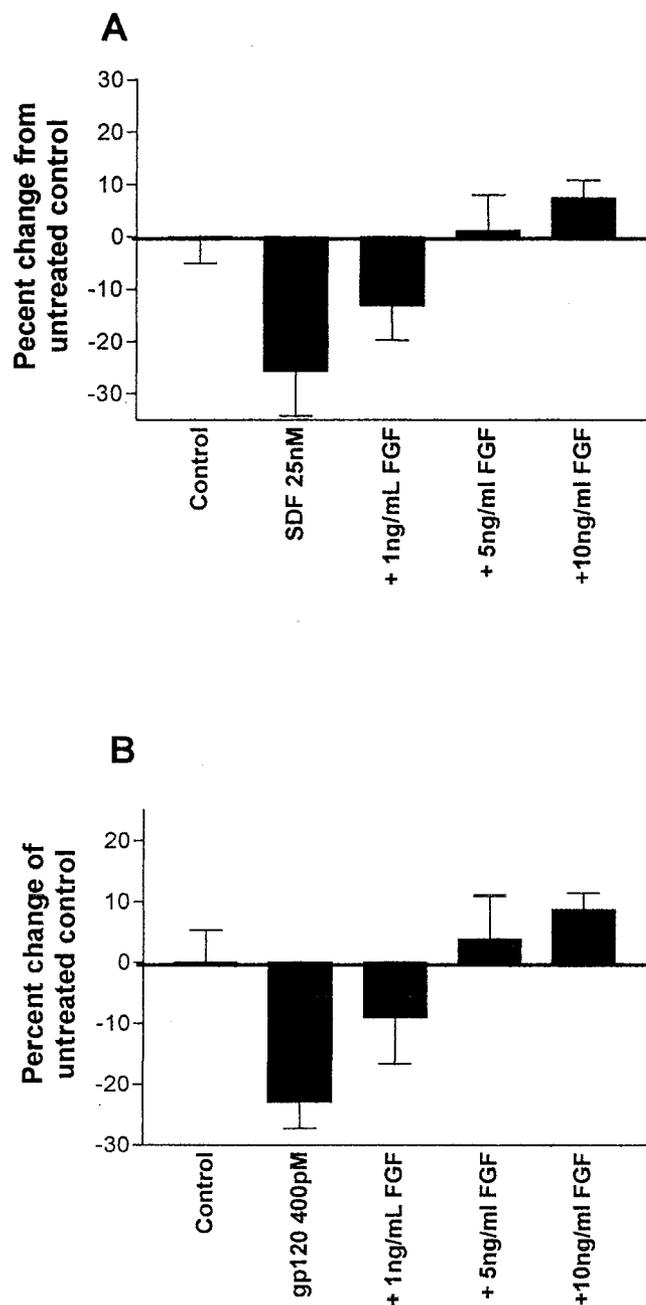


Fig. 4. FGF1 protects against stromal cell derived factor (SDF1) α - and human immunodeficiency virus (HIV) envelope protein gp120-induced neurotoxicity. Pretreatment with various concentrations of FGF1 increases cell survival in a dose-dependent manner of SH-SY5Y neuronal cells exposed to 25 nM SDF1 α (A) or 400 pm gp120IIIB (B). Cell survival was determined by lactate dehydrogenase release assay, and results are given as percent change of untreated control.

We measured protein levels of neuronal FGF and CXCR4 to determine whether there was an association similar to that observed in vitro. Consistent with those results, quantitative analysis of FGF1 and CXCR4 protein

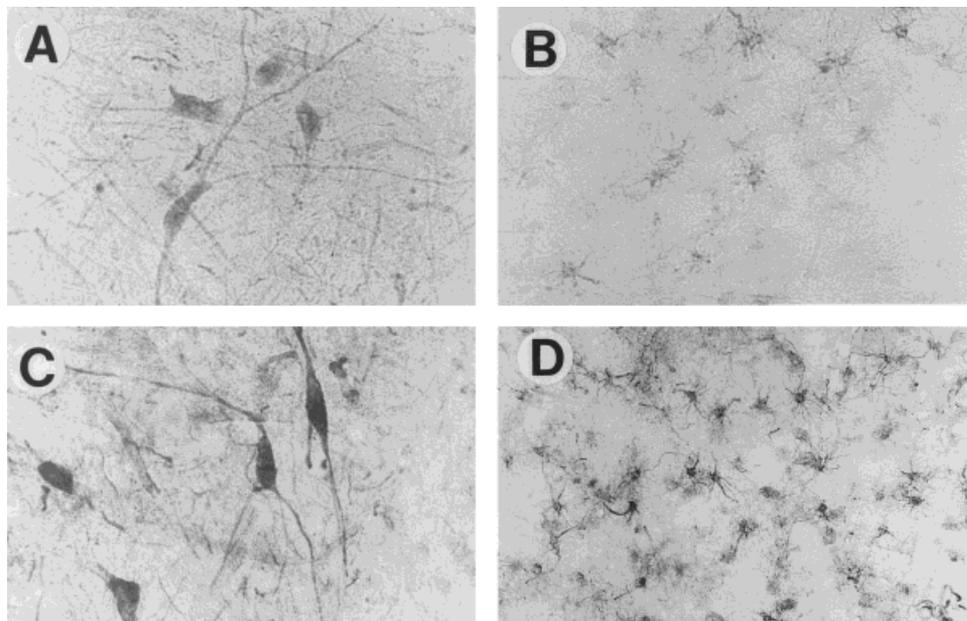


Fig. 5. Differential expression of neuronal FGF and CXCR4 is noted in brain tissue. **A:** FGF1 is localized to neuronal soma (400× magnification). **B:** FGF2 is localized to neuronal and astrocytic nuclei with occasional perinuclear and cytoplasmic staining (400×). **C:** CXCR4 is localized to neuronal soma (basal ganglia; 400×). **D:** In HIV encephalitis, cells with astrocytic morphology display intense immunoreactivity for CXCR4 (400×).

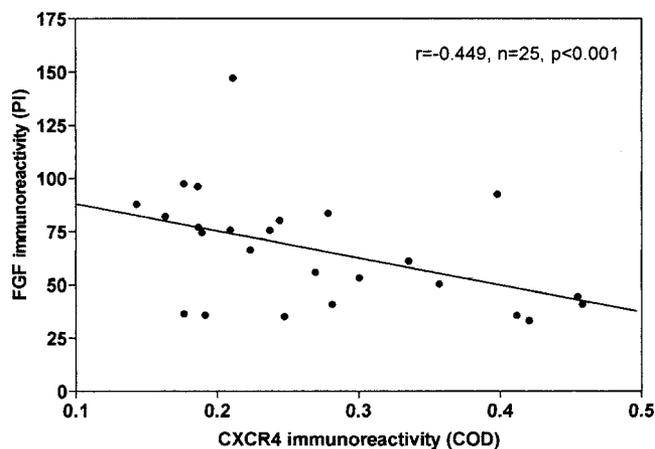


Fig. 6. High neuronal FGF immunoreactivity is associated with low neuronal CXCR4 immunoreactivity in the midfrontal cortex. Regression analysis of neuronal FGF immunoreactivity units given in fluorescent pixel intensity (PI), and neuronal CXCR4 immunoreactivity units given in corrected optical density (COD) reveal a significant negative association between FGF1 and CXCR4 levels.

levels as measured by pixel intensity and optical density, respectively, revealed a significant negative association between the two in the midfrontal cortex by regression analysis (Fig. 6). Higher FGF1 levels were associated with lower CXCR4 levels. Regression analysis between FGF1 and CXCR4 for basal ganglia neurons resulted in a similar but nonsignificant association (data not shown).

DISCUSSION

Fibroblast growth factor is known to promote cell proliferation and survival (Grothe and Wewetzer, 1996);

how it mediates these effects is under investigation. This study suggests mechanisms by which this growth factor may exert its effects by demonstrating FGF modulation of CXCR4 expression in the CNS. Furthermore, similar association of neuronal FGF and CXCR4 protein levels in HIVE suggest relevance to a disease process, while FGF’s ability to protect against SDF1 and gp120-induced neurotoxicity provides physiological relevance.

FGF1 downregulates neuronal CXCR4 in a dose-dependent manner. This type of FGF-induced receptor alteration has been reported by Mattson et al. (1995), who demonstrated that FGF protected against excitotoxicity by downregulating N-methyl-D-aspartate (NMDA) receptor protein levels after 12 hours of treatment. We saw maximal reduction of CXCR4 in 12 hours as well. Modulation of CXCR4 expression in neurons has not been thoroughly investigated. However, interferon-gamma has been reported to decrease receptor levels in endothelial cells (Gupta et al., 1998), astrocytes, and microglia (Gabuzda et al., 1999). Since CXCR4 signaling has been implicated in neuronal apoptosis, downregulation of the receptor by FGF would constitute a neuroprotective mechanism. To test this possibility, we measured cell survival of SH-SY5Y neuronal cells after exposure to SDF1α or gp120IIIB with or without pretreatment with FGF to reduce CXCR4 levels. Supporting our hypothesis, treatment of neuronal cells with FGF prior to exposure to toxic CXCR4 ligands increases cell survival in a dose-dependent manner.

FGF rapidly upregulates CXCR4 protein expression in astroglia. Consistent with this finding, prior studies have shown that FGF2 upregulates CXCR4 on human umbilical vein endothelial cells within 4 hours (Feil and Augustin, 1998). Other compounds upregulate expression as well. Interleukin (IL)-1β and tumor necrosis factor

(TNF) α induce upregulation of CXCR4 on microglia, and prostaglandin E₂ increases expression by astrocytes within 48 hours (Gabuzda et al., 1999). The stimulatory cytokine IL-4 and the glucocorticoid dexamethasone upregulate CXCR4 expression on T lymphocytes within 16 hours (Wang et al., 1998). The increase we observed occurred within 15 minutes, considerably more rapidly than the above reports. The FGF/endothelial cell study did not report a time course; it is possible that the effect on these cells could be seen at earlier times. It is also likely that astrocytes, cells known to respond to CNS injury in a short time, have the ability to upregulate CXCR4 protein expression in a more rapid manner. The bell-shaped response was an interesting and unexpected observation. FGF1 and 2 have two major high affinity receptors, FGFR1 (flg) and FGFR2 (bek), as well as having the ability to bind to low-affinity heparin sulfate proteoglycans. Higher concentrations of FGF may alter binding/signaling profiles by interacting with low-affinity sites and thus attenuate the increase of CXCR4 seen at lower concentrations. Taken together, these data suggest that CXCR4 protein expression is modulated by a number of factors and may play a significant role in astrocytic response to injury.

FGF displays opposite effects on glial and neuronal cells regarding CXCR4 expression; this observation may be explained by the broad pleiotrophic actions of FGF in response to injury. FGF is known to be involved in angiogenesis, astrocytosis, and neuroprotection. Differential regulation of FGF on CXCR4 protein levels demonstrated in this report for glia and neurons, as well as by others for endothelial cells (Feil and Augustin, 1998), would be one mechanism by which FGF exerts its effects. Astrogliosis and neovascularization would require an up-regulation of the receptor to promote chemoattraction to its ligand. Conversely, neuroprotection would require downregulation of neuronal CXCR4 if it acts to promote cell death.

We demonstrate that blocking FGF receptors with anti-receptor antibodies or MTA, the FGF receptor-specific tyrosine kinase inhibitor, attenuates the observed effects of FGF. This strongly suggests that FGF is acting via FGFR1 or FGFR2. However, it is unclear at this time which intracellular pathway is activated and results in CXCR4 transcriptional control. FGFR1 and FGFR2 have a number of different sites involved in substrate phosphorylation and subsequent diverging pathways (Szebenyi and Fallon, 1999). The Src family of kinases has been associated with FGF receptors and has been implicated in mediating FGF-induced migration as well as neuronal differentiation (Klint and Claesson-Welsh, 1999). Another potential pathway is via FRS2 and its downstream activation of Ras and microtubule-associated protein (MAP) kinases, which have also been implicated in neuronal differentiation and axonal growth (Derkinderen et al., 1999). Transcriptional control of CXCR4 has been investigated; a nuclear respiratory factor-1 (NRF-1) site as well as a TATA box and two GC boxes have been

identified. Interestingly, another region further upstream has been shown to inhibit promoter activity (Caruz et al., 1998; Wegner et al., 1998). Future studies will be directed at elucidating which pathway is involved in CXCR4 modulation.

In summary, this study demonstrates that fibroblast growth factor can decrease CXCR4 levels in neuronal cells and increase receptor levels in astroglial cells, both in a dose- and time-dependent manner. Since CXCR4 has been implicated in neuronal apoptosis directly or indirectly in the presence of gp120, FGF's reduction of chemokine receptor levels may play a neuroprotective role in HIV-induced neurodegeneration and thus may have therapeutic implications.

ACKNOWLEDGMENTS

The authors thank Dale Bredesen, La Jolla Cancer Research Institute, for providing the SH-SY5Y cells. This work was supported by National Institutes of Health grants MH12549-01 (V.J.S.), MH45294, MH59745, and DA12065 (E.M.).

REFERENCES

- Balaci L, Presta M, Ennas MG, Dell'Era P, Sogos V, Lauro G, Gremo F. 1994. Differential expression of fibroblast growth factor receptors by human neurones, astrocytes and microglia. *Neuroreport* 6:197-200.
- Caruz A, Samsom M, Alonso JM, Alcami J, Baleux F, Virelizier JL, Parmentier M, Arenzana-Seisdedos F. 1998. Genomic organization and promoter characterization of human CXCR4 gene. *FEBS Lett* 426:271-278.
- Davis CB, Dikic I, Unutmaz d, Hill CM, Arthos J, Siani MA, Thompson DS, Schlessinger J, Littman DR. 1997. Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. *J Exp Med* 186:1793-1798.
- Derkinderen P, Enslin H, Girault J. 1999. The ERK/MAP-kinases cascade in the nervous system. *NeuroReport* 10:R24-R34.
- Eckenstein FP, Kuzis K, Nishi R, Woodward WR, Meshul C, Sherman L, Ciment G. 1994. Cellular distribution, subcellular localization and possible functions of basic and acidic fibroblast growth factors. *Biochem Pharmacol* 47:103-110.
- Everall IP, DeTeresa R, Terry R, Masliah E. 1997. Comparison of two quantitative methods for the evaluation of neuronal number in the frontal cortex in Alzheimer disease. *J Neuropathol Exp Neurol* 56:1202-1206.
- Feil C, Augustin HG. 1998. Endothelial cells differentially express functional CXCR4-chemokine receptor-4 (CXCR4/fusin) under the control of autocrine activity and exogenous cytokines. *Biochem Biophys Res Commun* 247:38-45.
- Gabuzda D, Wang J, Ohagen A, He J. 1999. Chemokine receptors and mechanisms of cell death in HIV neuropathogenesis. HIV and the nervous system. Washington, DC: Emerging Issues. Section 4, page 7.
- Grothe C, Wewetzer K. 1996. Fibroblast growth factor and its implications for developing and regenerating neurons. *Int J Dev Biol* 40:403-410.
- Gupta SK, Lysko PG, Pillarisetti K, Ohlstein E, Stadel JM. 1998. Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines. *J Biol Chem* 273:4282-4287.
- Hesselgesser J, Taub D, Baskar P, Greenberg M, Hoxie J, Kolson DL, Horuk R. 1998. Neuronal apoptosis induced by HIV-1 gp120 and the chemokine SDF-1 alpha is mediated by the chemokine receptor CXCR4. *Curr Biol* 8:595-598.
- Himmelseher S, Pfenninger E, Georgieff M. 1996. The effect of basic

- fibroblast growth factor on glutamate-injured neuroarchitecture and arachidonic acid release in adult hippocampal neurons. *Brain Res* 707:54–63.
- Himmelseher S, Pfenninger E, Georgieff M. 1997. Effects of basic fibroblast growth factor on hippocampal neurons after axonal injury. *J Trauma* 42:659–664.
- Klint P, Claesson-Welsh L. 1999. Signal transduction by fibroblast growth factor receptors. *Front Biosci* 4:D165–177.
- Lavi E, Strizki JM, Ulrich AM, Zhang W, Fu L, Wang Q, O'Connor M, Hoxie JA, González-Scarano F. 1997. CXCR-4 (Fusin), a co-receptor for the type 1 human immunodeficiency virus (HIV-1), is expressed in the human brain in a variety of cell types, including microglia and neurons. *Am J Pathol* 151:1035–1042.
- Loetscher M, Geiser T, O'Reilly T, Zwahlen R, Baggiolini M, Moser B. 1994. Cloning of a human seven-transmembrane domain receptor, LESTR, that is highly expressed in leukocytes. *J Biol Chem* 269:232–237.
- Maggio R, Riva M, Vaglini F, Fornai F, Racagni G, Corsini GU. 1997. Striatal increase of neurotrophic factors as a mechanism of nicotine protection in experimental parkinsonism. *J Neural Transm* 104:1113–1123.
- Maher PA. 1993. Inhibition of the tyrosine kinase activity of the fibroblast growth factor receptor by the methyltransferase inhibitor 5'-methylthioadenosine. *J Biol Chem* 268:4244–4249.
- Mark RJ, Keller JN, Kruman I, Mattson MP. 1997. Basic FGF attenuates amyloid beta-peptide-induced oxidative stress, mitochondrial dysfunction, and impairment of Na⁺/K⁺-ATPase activity in hippocampal neurons. *Brain Res* 756:205–214.
- Masliah E, Mallory M, Hansen L, Alford M, DeTeresa R, Terry R, Baudier J, Saitoh T. 1992. Localization of amyloid precursor protein in GAP43-immunoreactive aberrant sprouting neurites in Alzheimer's disease. *Brain Res* 574:312–316.
- Mattson MP, Lovell MA, Furukawa K, Markesbery WR. 1995. Neurotrophic factors attenuate glutamate-induced accumulation of peroxides, elevation of intracellular CA²⁺ concentration, and neurotoxicity and increase antioxidant enzyme activities in hippocampal neurons. *J Neurochem* 65:1740–1751.
- Meucci O, Fatatis A, Simen AA, Bushell TJ, Gray PW, Miller RJ. 1998. Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc Nat'l Acad Sci USA* 95:14500–15405.
- Ohagen A, Ghosh S, He J, Huang K, Chen Y, Yuan M, Osathanondh R, Gartner S, Shi B, Shaw G, Gabuzda D. 1999. Apoptosis induced by infection of primary brain cultures with diverse human immunodeficiency virus type 1 isolates: evidence for a role of the envelope. *J Virol* 73:897–906.
- Sanders VJ, Pittman CA, White MC, Wang GJ, Wiley CA, Achim CL. 1998. Chemokines and receptors in HIV encephalitis. *AIDS* 12:1021–1026.
- Szebenyi G, Fallon JF. 1999. Fibroblast growth factors as multifunctional signaling factors. *Int Rev Cytol* 185:45–106.
- Wang J, Harada A, Matsushita S, Matsumi S, Zhang Y, Shioda T, Nagai Y, Matsushima K. 1998. IL-4 and a glucocorticoid up-regulate CXCR4 expression on human CD4⁺ T lymphocytes and enhance HIV-1 replication. *J Leuko Biol.* 64:642–649.
- Wegner SA, Ehrenberg PK, Chang G, Dayhoff DE, Sleeker AL, Michael NL. 1998. Genomic organization and functional characterization of the chemokine receptor CXCR4, a major entry co-receptor for human immunodeficiency virus type 1. *J Biol Chem* 273:4754–4760.
- Wirth SB, Rufer M, Unsicker K. 1996. Early effects of FGF-2 on glial cells in the MPTP-lesioned striatum. *Exp Neurol* 137:191–200.
- Yang SY, Cui JZ. 1998. Expression of the basic fibroblast growth factor gene in mild and more severe head injury in the rat. *J Neurosurg* 89:297–302.
- Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 393:595–599.