Brain-derived neurotrophic factor modulates expression of chemokine receptors in the brain

Farid Ahmed[1], **Lino Tessarollo**[2], **Carol Thiele**[3], and **Italo Mocchetti**[1]

1*Department of Neuroscience, Georgetown University Medical Center, Washington DC*

2*Neural Development Group, National Cancer Institute, Frederick, MD*

3*Paediatric Oncology Branch, National Cancer Institute, Bethesda MD*

Abstract

Chemokine receptors, and in particular CXCR4 and CCR5 play a key role in the neuropathogenesis of Human Immunodeficiency Virus-1 (HIV)4 associated dementia (HAD). Thus, new insight into the expression of CXCR4 in the central nervous system may help develop therapeutic compounds against HAD. Brain-derived neurotrophic factor (BDNF) is neuroprotective *in vitro* against two strains of the HIV envelope protein gp120 that binds to CXCR4 or CCR5. Therefore, we examined whether BDNF modulates chemokine receptor expression *in vivo*. The content of CXCR4 mRNA and proteins was determined in the cerebral cortex and hippocampus of 6-month-old BDNF heterozygous mice and wild type littermates by using polymerase chain reaction and immunohistochemistry, respectively. BDNF heterozygous mice exhibited an increase in CXCR4 mRNA compared to wild type. Histological analyses revealed an up-regulation of CXCR4 immunoreactivity mainly in neurons. Most of these neurons were positive for TrkB, the BDNF receptor with a tyrosine kinase activity. Increases in CXCR4 mRNA levels were observed in 18-month-old BDNF heterozygous mice but not in 7 day-old mice, suggesting that the modulatory role of BDNF occurs only in mature animals. To determine whether BDNF affects also CXCR4 internalization, SH-SY5Y neuroblastoma cells were exposed to BDNF and cell surface CXCR4 levels were measured at various times. BDNF induced CXCR4 internalization within minutes. Lastly, BDNF heterozygous mice showed higher levels of CCR5 and CXCR3 mRNA than wild type in the cerebral cortex, hippocampus and striatum. Our data indicate that BDNF may modulate the availability of chemokine receptors implicated in HIV infection.

Keywords

BDNF; HIV dementia; CXCR4; CCR5; gp120; TrkB

1. Introduction

Chemokine and chemokine receptors were originally identified for their chemoattractant and pro-adhesive roles in inflammation, wound repair, and cell migration [rev in (Miller and Tran, 2005)]. During the development of the central nervous system (CNS), the chemokine receptor CXCR4 plays a crucial role in the migration of cerebrocortical interneurons (Stumm et al., 2003) as well as granule cells of the cerebellum (Ma et al., 1998) and the hippocampal dentate gyrus (Bagri et al., 2002; Lu et al., 2002). Indeed, the lack of CXCR4 results in an improper formation of neuronal layers in these areas. However, CXCR4 is also abundant in the adult CNS. For instance, pyramidal cells of the cortex, dopaminergic neurons of the substantia nigra and cholinergic neurons of the basal forebrain are especially enriched in CXCR4 in rats (Banisadr et al., 2002). Astrocytes and microglia cells of different brain regions also express CXCR4 (Banisadr et al., 2002; Lavi et al., 1997) even in the absence of inflammatory responses (Lazarini et al., 2003). Thus, the anatomical profile and abundance of CXCR4 and other chemokines in the adult CNS suggest a broader function than the originally proposed neuroinflammation and migration role.

CXCR4 has recently been recognized as crucial mediators of Human Immunodeficiency Virus-1 (HIV) entry into immunocompetent target cells [rev in (Moore et al., 2004)]. Sequential binding of the HIV envelope glycoprotein 120 (gp120) to CD-4 and chemokine receptors on the surface of immunocompetent cells facilitates HIV entry into cells (Berson et al., 1996; Feng et al., 1996; He et al., 1997). Different strains of gp120 exhibit affinity for specific chemokine receptors differentially expressed on certain cell types. For example, gp120 associated with M-tropic HIV binds specifically to CCR5, which is expressed on macrophages, whereas gp120 associated with T-tropic HIV binds to CXCR4, which is abundant in T-cells (Moore et al., 2004). Thus, availability of chemokine receptors is crucial for the infectious phase of the disease.

There is also growing evidence for the role of chemokines in the neurotoxicity of HIV in the CNS. Neurons are susceptible to massive apoptotic cell death in the presence of T-tropic gp120 or other viral proteins both *in vitro* and *in vivo* (Bansal et al., 2000; Kaul and Lipton, 1999; Kruman et al., 1998; Meucci et al., 1998; Nosheny et al., 2004). Moreover, mice overexpressing T-tropic gp120 (Toggas et al., 1994) exhibit several features that mirror the neuropathology of HAD. The neurotoxic effect of gp120 involves CXCR4-mediated activation of pro-apoptotic signal transduction cascades, including caspase-3 (Bachis et al., 2003; Biard-Piechaczyk et al., 2000; Hesselgesser et al., 1998; Zheng et al., 1999). These results imply a fundamental role for CXCR4 in HIV-Associated Dementia (HAD) pathogenesis, which includes neuronal apoptosis and atrophy (Gray et al., 2001; James et al., 1999; Masliah et al., 2000; McArthur, 2004), and suggest that targeted therapeutic interventions for HAD should include a down-regulation of chemokine receptor function.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of trophic factors that includes nerve growth factor, neurotrophin-3 and NT4/5 (Chao, 2003). There is evidence that BDNF reduces the neurotoxicity of gp120 *in vitro* (Bachis and Mocchetti, 2005) and *in vivo* (Nosheny et al., 2007). The neuroprotective effect correlates with its ability to reduce CXCR4 levels *in vitro* (Bachis et al., 2003). However, little is known about the modulation of CXCR4 *in vivo*. Moreover, little is known about the ability of BDNF to induce CXCR4 internalization. In the present study, we used BDNF heterozygous (+/−) mice as well as *in vitro* studies in an attempt to characterize a relationship between BDNF and modulation of chemokine receptor availability. Such details about BDNF activity may provide an avenue for better treatment or prevention of HAD.

2. Results

BDNF heterozygous mice exhibit altered levels of CXCR4 mRNA

To examine the role of BDNF in regulating CXCR4 expression *in vivo* we utilized 6-month-old BDNF$^{+/-}$ mice and age-matched wild type (WT) littermates. One may predict that low levels of BDNF, as in those exhibited by BDNF$^{+/-}$ mice (Lyons et al., 1999), will cause CXCR4 up-regulation. CXCR4 mRNA was measured using a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and specific primers for mouse CXCR4 (Table 1). Primers for ribosomial protein 19 (RPL19) were used as an internal control to determine relative levels of total mRNA and standardize CXCR4 mRNA levels. We found that BDNF$^{+/-}$ mice showed higher CXCR4 mRNA levels than WT in the cerebral cortex (Fig. 1A), hippocampus and striatum (Fig. 1B), while the levels of RPL19 mRNA remained unchanged. No differences in CXCR4 mRNA were found between WT and heterozygous mice in the hypothalamus and cerebellum (Fig. 1B), the two areas with highest levels of CXCR4 mRNA.

Changes in CXCR4 immunoreactivity in heterozygous mice occur in neurons

Both neuronal and non-neuronal cells express CXCR4 (Banisadr et al., 2002; Lavi et al., 1997; Lazarini et al., 2000; Westmoreland et al., 2002). We therefore used histological analyses to examine the relationship between low levels of BDNF and cells expressing CXCR4. We first analyzed the cerebral cortex and hippocampus of 6-month-old WT mice for CXCR4 immunoreactivity, using a CXCR4 specific antibody together with Nissl, glial fibrillary acidic protein (GFAP) and CD11 staining to detect neurons, astrocytes and microglia, respectively. CXCR4 positive and negative cells were observed throughout the cerebral cortex (Figs. 2A and B). Specificity of the antibody was confirmed by omitting the primary (Fig. 2C) or secondary antibody (Fig. 2D). In both cases, no CXCR4 immunoreactivity was detected (Figs 2C and D). Most of CXCR4 immunoreactivity was detected in several Nissl positive cells in layers II/III (data not shown) and V (Figs. 2E and G). Some Nissl positive cells were CXCR4 negative (Figs. 2E and G, arrows). Occasionally, we observed CXCR4 immunoreactivity in Nissl negative cells (Figs. 2F and G, arrowhead).

In the hippocampus, CXCR4 immunoreactivity was seen in neurons as well as in astrocytes (Fig. 2H), supporting previous data that in adult rats several cell types exhibit CXCR4 immunoreactivity (Banisadr et al., 2002). Sections from WT and BDNF$^{+/-}$ mice were then used to compare the number of CXCR4 positive cells. The number of CXCR4 positive cells was determined in layer V of the frontal cortex and the dorsolateral portion of the hippocampus corresponding to the CA2 area. Semi-quantitative analysis using Metamorph® revealed that more neurons were positive for CXCR4 in BDNF$^{+/-}$ mice than in WT in both regions (Fig. 3). There was no difference in CXCR4 positive astrocytes between WT and heterozygous mice. Moreover, no CD11 immunoreactivity (microglia) was seen in either WT or BDNF$^{+/-}$ mice (data not shown).

TrkB positive neurons exhibit CXCR4 immunoreactivity

BDNF has been shown to prevent gp120 neurotoxicity by activating the tyrosine kinase receptor TrkB (Mocchetti and Bachis, 2004). Therefore, we used a TrkB specific antibody (TrkB$_{23-36}$) developed against the extracellular portion of TrkB (Fryer et al., 1996) to provide a correlation between distribution of the TrkB that signals in response to BDNF and CXCR4 expression. This antibody has been shown to stain neurons in the cortex and hippocampus (Fryer et al., 1996). In the cortex (Figs. 4A–C) and hippocampus (data not shown) of WT animals, most of TrkB positive cells exhibited CXCR4 immunoreactivity. To distinguish whether these neurons expressed full-length or truncated forms of TrkB, adjacent sections were stained with an antibody that recognizes the tyrosine kinase intracellular portion of Trk (TrkB-

in). All neurons that were stained with $TrkB_{23-36}$ exhibited the same immunoreactive profile for TrkB-in (not shown), suggesting that these neurons express the full-length TrkB.

Neurons are the major source of TrkB immunoreactivity in layer V of the cortex. However, few astrocytes were detected in this layer. To assure the neuronal expression of TrkB, $TrkB_{23-36}$ was used in conjunction with a GFAP antibody in co-localization studies of hippocampal sections. TrkB immunoreactivity was mostly detected in the dentate gyrus (Fig. 4H), and CA regions of the hippocampus (data not shown) confirming previous data (Fryer et al., 1996). GFAP positive cells were TrkB negative (Fig. 4G–I). Because CXCR4 immnoreactivity was also detected in astrocytes, our data overall suggest that CXCR4 does not always co-localize with TrkB.

Sections from WT and $BDNF^{+/-}$ mice were then used to compare the number of CXCR4 positive cells in TrkB positive/negative neurons. In the cortex, more CXCR4/TrkB positive cells were observed in $BDNF^{+/-}$ animals (Figs. 4D–F) than WT (Figs. 4A–C). However, the total number of TrkB positive neurons was similar in both animal groups (Figs. 4B and E). In the hippocampus, more CXCR4 immunoreactivity was observed in $BDNF^{+/-}$ mice (Fig. 3), but no changes were observed in GFAP (Fig. 4J) or TrkB positive cells (Fig. 4K), suggesting that the observed changes in CXCR4 positive neurons in $BDNF^{+/-}$ mice are not due to alterations of cell number. Overall, these results support previous pharmacological data that TrkB plays a crucial role in the regulation of CXCR4 expression (Mocchetti and Bachis, 2004).

CXCR4 mRNA up-regulation is age-dependent

$BDNF^{+/-}$ mice exhibit an age-dependent reduction of fibers and function of central serotonergic neurons (Lyons et al., 1999). To determine whether BDNF regulation of CXCR4 expression is age-dependent, cortical CXCR4 mRNA levels were measured by PCR (Fig. 5A) in 7-day-old pups, 6- and 18-month-old mice. In WT, there was no significant difference in CXCR4 mRNA levels between 7- day and 6- and 18-month-old mice (Fig. 5), suggesting that maximal expression of CXCR4 occurs already a few days postnatal. Interestingly, while 18-month-old $BDNF^{+/-}$ mice exhibited a ~two-fold increase in CXCR4 mRNA levels (Fig. 5B), 7-day-old WT and $BDNF^{+/-}$ displayed the same amount of CXCR4 mRNA levels (Fig. 5). Thus, BDNF appears to regulate CXCR4 expression only in adulthood.

BDNF reduces membrane associated CXCR4

CXCR4 is a G protein-coupled receptor (GPCR) that undergoes ligand induced internalization (Tarasova et al., 1998). In neurons, internalization of gp120 and retrograde axonal transport are necessary for the toxic activity of gp120 (Bachis et al., 2003; Bachis et al., 2006). Therefore, we examined whether BDNF alters membrane-associated CXCR4. Internalization of CXCR4 by BDNF was determined by cell surface biotinylation. Because this assay requires several micrograms of proteins that cannot be easily obtained from primary neuronal cultures, we used human SH-SY5Y neuroblastoma cells. These cells are sensitive to the toxic action of T tropic gp120 (Bardi et al., 2006). However, SH-SY5Y lacks endogenous expression of trkB (Kaplan et al., 1993). Thus, we used SH-SY5Y cells over-expressing TrkB (clone TB8) under a tetracycline-regulated promoter (Kim et al., 1999). These cells, in addition to be BDNF responsive, differentiate into a neuronal phenotype (Kim et al., 1999). Cells were exposed to medium control or containing BDNF (10 ng/ml) for various times (5, 30 min and 3 hr). Cells were then incubated with membrane impermeant NHS-LC-Biotin for 30 min at 4°C, and biotinylated cell surface proteins were precipitated with immobilized streptavidin and subjected to CXCR4 immunoblotting. Immobilized streptavidin precipitated a complex of CXCR4 immunoreactivity of approximately 42 kDa (Fig. 6A), which is consistent with the

molecular weight of CXCR4. In cells exposed to BDNF, CXCR4 immunoreactivity decreased within 5 min of exposure (Fig. 6B), suggesting increased internalization.

To determine whether BDNF affects also the expression of CXCR4, SY5TB8 cells were exposed to BDNF (10 ng/ml) for 4 and 8 hr. CXCR4 mRNA levels were then measured by RT-PCR using primers designed to amplify human CXCR4 mRNA (Table 1). The human housekeeping gene cyclophilin was used as an internal control to standardize CXCR4 mRNA levels. BDNF promoted a decrease in CXCR4 mRNA at both 4 and 8 hr (Fig. 7). This data, together with previous results showing reduced CXCR4 protein levels in cerebellar granule cells exposed to BDNF (Bachis et al., 2003), indicates that this trophic factor modulates CXCR4 expression and trafficking.

CCR5 and CXCR3 mRNA levels are up-regulated in BDNF +/− mice

HIV-1 can generally use CCR5 as co-receptors for cell entry (Signoret et al., 2005). In addition, CXCR3 appears to mediate the toxic action of CXCR4 ligands (Vergote et al., 2006). Therefore, it is important to establish whether BDNF alters the expression profile of these chemokine receptors. Total RNA was extracted from the cerebral cortex, hippocampus and striatum of 6-month-old mice and analyzed by RT-PCR (Fig. 8A). BDNF$^{+/−}$ mice displayed higher CXCR3 and CCR5 mRNA levels than WT in all regions examined (Fig. 8B). However, the hippocampus of BDNF+/− mice exhibited a robust 7-fold increase in CCR5 mRNA levels (Fig. 8C). Thus, chemokine receptor expression by BDNF follows a region-specific pattern of regulation.

3. Discussion

A number of HIV positive individuals develop HAD, which is characterized pathologically by neuronal loss and dysfunction (Everall et al., 2005). HIV infection is mediated by the co-receptors CXCR4 and CCR5. Thus, in this report, we investigated whether chemokine receptor expression can be regulated in the brain. We used BDNF$^{+/−}$ mice because BDNF exhibits neuroprotection *in vitro* against two strains of gp120 that binds to CXCR4 and CCR5 (Bachis and Mocchetti, 2005). Moreover, BDNF$^{+/−}$ mice are more sensitive to gp120 toxicity (Nosheny et al., 2004). In these animals, we found an increase of CXCR4, CCR5 and CXCR3 mRNAs in several brain areas and an accumulation of CXCR4 immunoreactivity in neurons. Previous data have shown that BDNF down-regulates CXCR4 levels *in vitro* (Bachis et al., 2003) and *in vivo* (Nosheny et al., 2007). Moreover, we show that in SY5YTB8 neuroblastoma cells, BDNF accelerates CXCR4 trafficking and reduces CXCR4 mRNA levels. These data, taken together, indicate that BDNF modulates expression and availability of chemokine receptors both physiologically and pharmacologically. Based on these findings, we propose that down-regulation of CXCR4 is a mechanism that may account for the neuroprotective property of BDNF against gp120.

CXCR4 immunoreactivity has been detected in neurons as well as non-neuronal cells, including microglia and astrocytes in numerous brain areas of different species (Banisadr et al., 2002; Klein et al., 1999; Lazarini et al., 2003; van der Meer et al., 2000). In this report, we confirm that neurons and astrocytes express CXCR4. However, BDNF appears to affect CXCR4 immunoreactivity only in neurons. Indeed, in BDNF$^{+/−}$ mice we observed an increase in CXCR4 positive neurons in both the cortex and hippocampus without a concomitant increase in CXCR4 immunoreactivity in astrocytes. This result is not surprising because neurons are known to express catalytically active TrkB (Fryer et al., 1996), the receptor that mediates the neurotrophic activity of BDNF, including its neuroprotective effect against gp120 (Mocchetti and Bachis, 2004). Indeed, in our study, astrocytes were not labeled by a TrkB specific antibody (Fryer et al., 1996), while a number of neurons in layer V of the cortex, dentate gyrus and CA regions of the hippocampus were both CXCR4 and TrkB positive. Thus, BDNF may not

regulate CXCR4 expression in non-neuronal cells because of the absence of TrkB. These data support previous pharmacological results showing that TrkB is the main neurotrophin receptor that, by decreasing CXCR4 levels, modulates the neuroprotective property of BDNF (Mocchetti and Bachis, 2004).

BDNF$^{+/-}$ mice exhibited an increase in CXCR4 mRNA content in the cerebral cortex, hippocampus and striatum. We were surprised to find that the content of CXCR4 mRNA in the hypothalamus and cerebellum of BDNF$^{+/-}$ mice was similar to that of WT. The reason of this anatomically-restricted effect is unclear at present. Both the cerebellum and hypothalamus express TrkB (Givalois et al., 2004; Ohira and Hayashi, 2003). Nevertheless, in these areas, CXCR4 may not be co-localized with TrkB. Our data have shown that astrocytes can be CXCR4 positive but TrkB negative. Moreover, glial cells express the truncated isoform of TrkB (Fryer et al., 1996), which does not appear to mediate most of the neurotrophic effect of BDNF; rather this receptor prevents BDNF signaling (Dorsey et al., 2006). Only detailed anatomical studies will reveal why BDNF has a restricted action on CXCR4 in these areas. On the other hand, both cerebellum and hypothalamus exhibit the highest levels of CXCR4 mRNA among all regions examined, suggesting that the expression of this receptor may be influenced by other stimuli. Defining the molecular mechanisms of this regulation may help a better understanding of the role of CXCR4 in adult brain.

BDNF down-regulation of CXCR4 expression is believed to underlie its neuroprotective property against gp120 (Bachis et al., 2003; Nosheny et al., 2007). A similar cellular mechanism for neuroprotection has been proposed for another growth factor, basic fibroblast growth factor (FGF2), a member of the heparin binding growth factor that has neuroprotective properties similar to BDNF. In fact, FGF2 limits gp120 and SDF toxicity in SH-SY5Y neuroblastoma cells by reducing CXCR4 levels (Sanders et al., 2000). Most of the neuroprotective activities of BDNF and FGF2 occur through the activation of high affinity tyrosine kinase receptors TrkB and FGF receptor 1, respectively. Both receptors share a similar signal transduction mechanism. BDNF or FGF2 binding to TrkB or FGF receptor leads to activation of a number of target proteins including phosphatidylinositol-3 kinase, phospholipase c-γ, and extracellular signal regulated kinase (Miller and Kaplan, 2003). Thus, it is not surprising to observe a similar and perhaps overlapping neuroprotective profile between these two trophic factors. Indeed, blocking signaling of either TrkB by K252a (Mocchetti and Bachis, 2004) or FGF receptor by 5'-methylthioadenosine (Sanders et al., 2000) abolishes the neuroprotective effect of BDNF and FGF2, respectively. The fact that both trophic factors inhibit gp120 and SDF toxicity by reducing CXCR4 levels adds to the notion that modulation of CXCR4 is a viable strategy to limit HIV-mediated apoptosis.

In this report, we utilized BDNF$^{+/-}$ mice to analyze the effect of BDNF on CCR5 and CXCR3 mRNA levels. CCR5 is another co-receptor used by HIV for cell entry (Signoret et al., 2005). CXCR3 mediates the toxic action of CXCR4 ligands (Vergote et al., 2006). Both CXCR3 and CCR5 mRNA levels were increased in BDNF$^{+/-}$ mice in the cerebral cortex, hippocampus and striatum. Moreover, we observed an age-dependent increase in CXCR4 mRNA levels in these animals. Intriguingly, while the net increase in CXCR3 and CXCR4 mRNAs was similar in all regions examined, CCR5 mRNA levels in the hippocampus exhibited the highest increase. The physiological significance of this effect is unclear. In the adult hippocampus, CCR5 expression is mainly localized in neurons of the CA1 region and dentate gyrus (Torres-Munoz et al., 2004; Westmoreland et al., 2002) and within neural progenitor cells (Tran et al., 2007). Multipotent neural progenitor cells are still present in the adult hippocampus and are capable of giving rise to functional neurons and glial cells (Lledo et al., 2006). BDNF has a crucial role in hippocampal neurogenesis (Schmidt and Duman, 2007). Therefore, it may be worth speculating that a balance between BDNF and chemokine receptors may contribute to modulate adult neurogenesis.

Molecular and cellular mechanisms of neuroprotection against HAD are crucial for the development of new adjunct therapies. In this and other reports (Bachis et al., 2003; Nosheny et al., 2007), we have provided evidence that BDNF regulates the expression of chemokine receptors implicated in HIV infection. Therefore, one may speculate that BDNF, by reducing membrane associated CXCR4, may help diminish the neurotoxic effect of T-tropic virus. These findings raise hope for a pharmacological intervention in HAD in adjunction to the current antiviral therapy.

4. Experimental Procedures

Animals

7-day, 6-month and 18-month-old $BDNF^{+/-}$ mice and WT littermates were generated in the C57BL/6 genetic background as previously described (Lyons et al., 1999) and were back-crossed for 10–12 generations to a C57BL/6 genetic background, a strategy resulting in great reduction of the genetic heterogeneity present in the original 129 Sv-C57BL/6 mixed background. Mice were group-housed under standard conditions, three to five per cage with food and water available *ad libitum* and were maintained on a 12-hr light/dark cycle. Both male and female mice were used for the analyses.

Cell cultures

SH-SY5Y neuroblastoma cells over-expressing TrkB (clone TB8) were prepared and grown as previously described (Kim et al., 1999). Cells were plated onto precoated 100 mm dishes (Corning Inc., Corning, NY) and grown in RPMI 1640 (Cambrex Bio Science, Walkersville, MD) containing 10% fetal bovine serum, 2 mmol/L glutamine and 0.5 μg/ml puromycin.

Semi-Quantitative RT-PCR

Total RNA was isolated from brain areas or SY5YTB8 cells using absolute RNA PCR isolation kit (Strategene, La Jolla, CA) according to the manufacturer's protocol. Total RNA (5μg) was treated with RNAse-free DNAse and then reverse-transcribed using SuperScript and oligo(dT)-primer (Invitrogen, Grand Island, NY) according to the manufacturer's protocol in 20 μl of reaction mixture. The resulting cDNA was amplified by PCR using sense and antisense primers described in Table 1.

PCR reactions were performed in a Genius thermal Cycler using GoTag green master mix (Promega Corp. Madison, WI). The thermal cycling parameters were as follows: 1 min 30 sec at 94°C followed by 25 cycles (housekeeping gene RPL19 and cyclophilin) and 30 cycles (CXCR3, CXCR4 and CCR5) of 30 sec at 94°C, 1 min 30 sec at 59°C, 1 min at 72°C and final incubation for 5 min at 72°C. Different dilutions of cDNA samples were used for different genes to provide a linear range of PCR reactions. The products were separated on a 2% agarose gel in Tris/acetate/EDTA buffer containing ethidium bromide. Relative abundance of mRNAs for chemokine receptors was estimated based on the intensity of cDNA bands using Quantity One 1-D Analysis Software (BioRad, Lab., Inc. Hercules, CA).

Immunohistochemistry

Mice were anesthetized with 2-2-2-tribromoethanol (125 mg/kg, Sigma) and perfused transcardially with 4% paraformaldehyde. The brain was removed and post-fixed in 4% paraformaldehyde, then transferred into buffered graded sucrose (10%, 20% and 30%) and serial cross sections (16 μm) throughout the somatosensory cortex were prepared. For CXCR4 and Nissl, sections were incubated for 24 hr at 4°C with a CXCR4 monoclonal antibody (5 μg/ml, NIH AIDS Research Reference Reagent Program, Rockville, MD) followed by fluorescein-conjugated secondary antibody (Alexafluor 546, cat #A-11030, 1:500 dil, Molecular Probes,

Eugene, OR) for 1 hr at room temperature to visualize CXCR4. Sections were then incubated with NeuroTrace™ green fluorescent Nissl Stain (cat # N21489, 1:100 dil, Molecular Probes). For CXCR4 and TrkB, sections were incubated for 24 hr at 4°C with CXCR4 antibody as above, together with a polyclonal TrkB antibody (αTrkB$_{23-36}$ or TrkB-in, 1:200 dilution, a gift from Dr. D.R. Kaplan, Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada). The characterization of these antibodies has been described elsewhere (Fryer et al., 1996). Alexafluor 546 (Molecular probe) and FITC (1:100, cat# F7367, Sigma St. Louis, MO) were used as secondary antibodies. For CXCR4 and GFAP, sections were incubated with the CXCR4 antibody described above for 24 hr at 4°C together with polyclonal GFAP antibody (4 µg/ml, Advanced Immunochemical, Long Beach, CA) followed by corresponding secondary antibodies FITC or Cy3, (cat # 706-165-148, 1:100 dil, Jackson ImmunoReserach Lab., West Grove, PA). For TrkB and microglia, sections were incubated with the TrkB antibody as above together with a monoclonal CD11b antibody (10 µg/ml, Serotec, Raleight, NC) followed by corresponding secondary antibodies FITC and Alexafluor 546. All primary and secondary antibodies were diluted in blocking reagent (0.01% triton, 2% BSA in 1× PBS). Reaction was visualized with a Zeiss fluorescence microscope Axiophot2. Sections were examined using a 20X objective. Total intensity of fluorescent signal was determined by using MetaMorph® software (Universal Imaging Corporation™, Downingtown, PA) as previously described (Bachis et al., 2003; Nosheny et al., 2004). A total of 10 sections (1 section every 80 µm) per animal was used (N=4 mice per group).

Cell surface biotinylation and streptavidin precipitation

Cell surface proteins were isolated using Pinpoint Cell Surface Protein Isolation Kit (Pierce, Rockford, IL) according to the manufacturer's protocol. In brief, the day of the experiment, SY5YTB8 (Kim et al., 1999) were exposed to human recombinant BDNF (Amgen Inc., Thousand Oaks, CA), for various time points at 37°C. Medium was then replaced by PBS and cells were incubated with sulfo-NHS-LC-biotin (2 mM; Pierce, Rockford, IL) in PBS for 30 min at 4°C. Cells were then harvested and centrifuged at 500×g for 3 min and the pellet was washed once by adding 5 ml of TBS. Cells were lysed using a lysis buffer containing protease inhibitor cocktail (P 8340 Sigma, Saint Louis, MO), for 30 min at 4°C. After removal of insoluble proteins, supernatants were incubated for 1 hr at room temperature in a column containing 500 µl of immobilized neutravidin gel slurry. The biotin–streptavidin–agarose complexes were collected after centrifugation and washed three times in wash buffer. The complexes were then incubated with SDS-PAGE sample buffer, 50 mM DTT for 1 hr at room temperature. Biotinylated surface protein was finally isolated by 2 min centrifugation at 1000× g. Proteins were loaded into a 4–12% SDS-PAGE gel, and blots were probed with a CXCR4 antibody (1:1000, Santa Cruz Biotec., Inc., Santa Cruz, CA).

Data analysis

Statistical analysis was done by ANOVA followed by Student's t test or ANOVA and Scheffe's test for multiple comparisons (SigmaStat, SPSS Science, San Raphael, CA).

Acknowledgements

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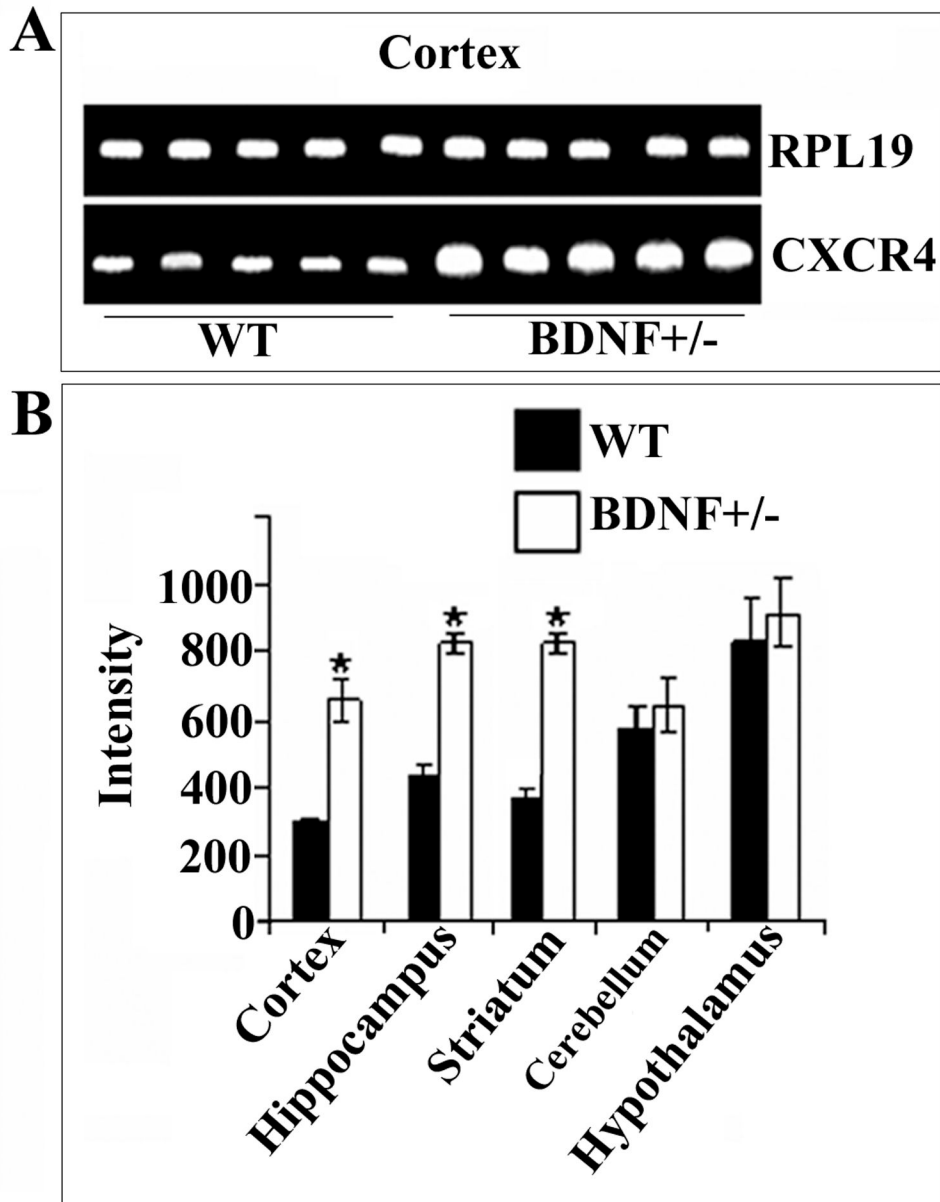
Figure 1. BDNF heterozygous mice exhibit higher levels of CXCR4 mRNA than WT
RNA was extracted from the indicated brain areas of 6-month-old BDNF$^{+/-}$ mice or WT littermates. RT-PCR was performed using primers designed to amplify mouse CXCR4 (see Experimental Procedures). PCR reaction products were analyzed by agarose gel-electrophoresis (see Experimental Procedures). **A**. Representative gel showing CXCR4 and RPL19 cDNAs from the cerebral cortex. **B**. Semi-quantitative analysis of CXCR4 cDNA was carried out by Quantity One 1-D Analysis as described in Experimental Procedures. RPL19 was used as an internal control to normalize gel loading. Data, expressed as intensity of the cDNA band, are the mean ± SEM of five independent samples. *p<0.05 vs control.
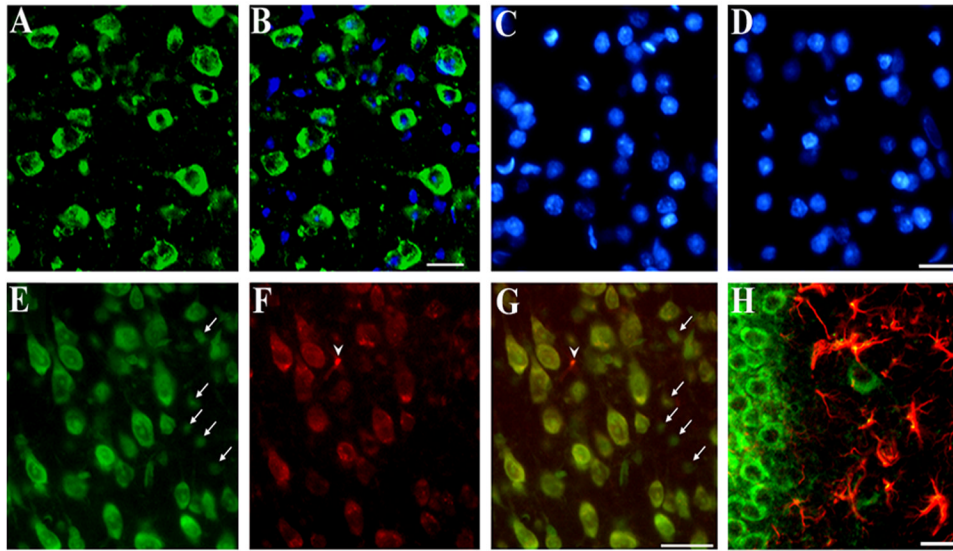
Figure 2. Immunohistochemical analysis of CXCR4
Serial coronal sections (16 µm) from the frontal cortex or hippocampus were obtained from WT mice. Examples of cortical sections stained with (**A**) a CXCR4 antibody (green) counterstained with (**B**) DAPI (blue) to visualize cellular nuclei. Note that some cells are CXCR4 negative. **C and D:** Examples of sections in which the primary or secondary antibody, respectively, were omitted. Sections were counterstained with DAPI. **E and F:** Sections stained with Nissl and CXCR4 antibody, respectively (see Experimental Procedures). **G:** E and F were merged to show colocalization of CXCR4 and neurons (yellow). Note that in E small Nissl positive cells are CXCR4 negative (arrows). In F, one CXCR4 positive cell (arrowhead) is Nissl negative. **H:** Example of a hippocampal section stained with CXCR4 (green) and GFAP (red) and showing colocalization of CXCR4 and GFAP (yellow). Bars=40 µm.
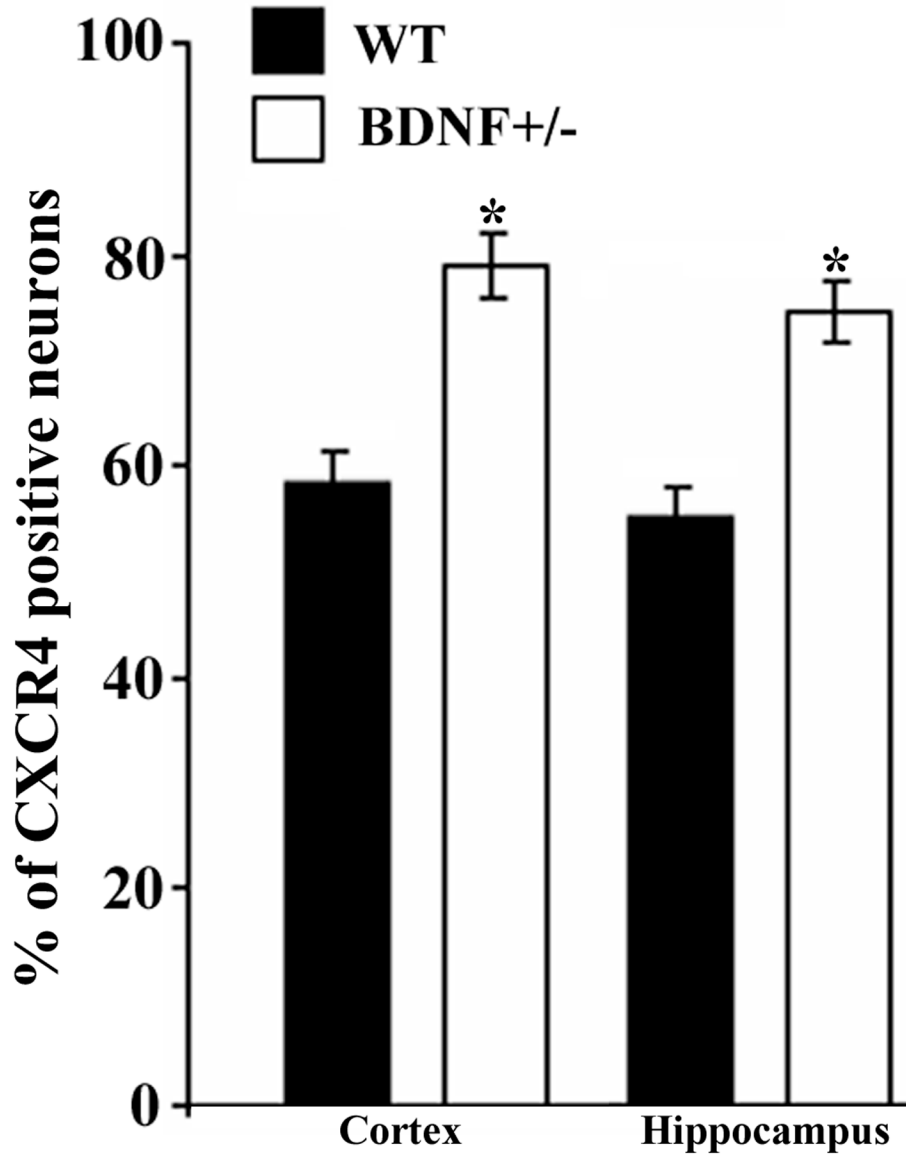
Figure 3. BDNF$^{+/-}$ mice exhibit more CXCR4 immunoreactivity
Serial coronal sections obtained as described in Fig. 2 and in Experimental Procedures were used to determine the number of CXCR4 positive neurons in the frontal cortex and hippocampus. The number of CXCR4 positive neurons was determined in layer V of the cortex and CA2 region of the hippocampus in an area of 1 mm^2 per section using MetaMorph® software. An average of 8000 neurons per animal per area was counted. Data, expressed as % of Nissl positive cells per section, are the mean ± SEM of 10 sections per animal (n=4 each group). *p<0.05 vs WT.
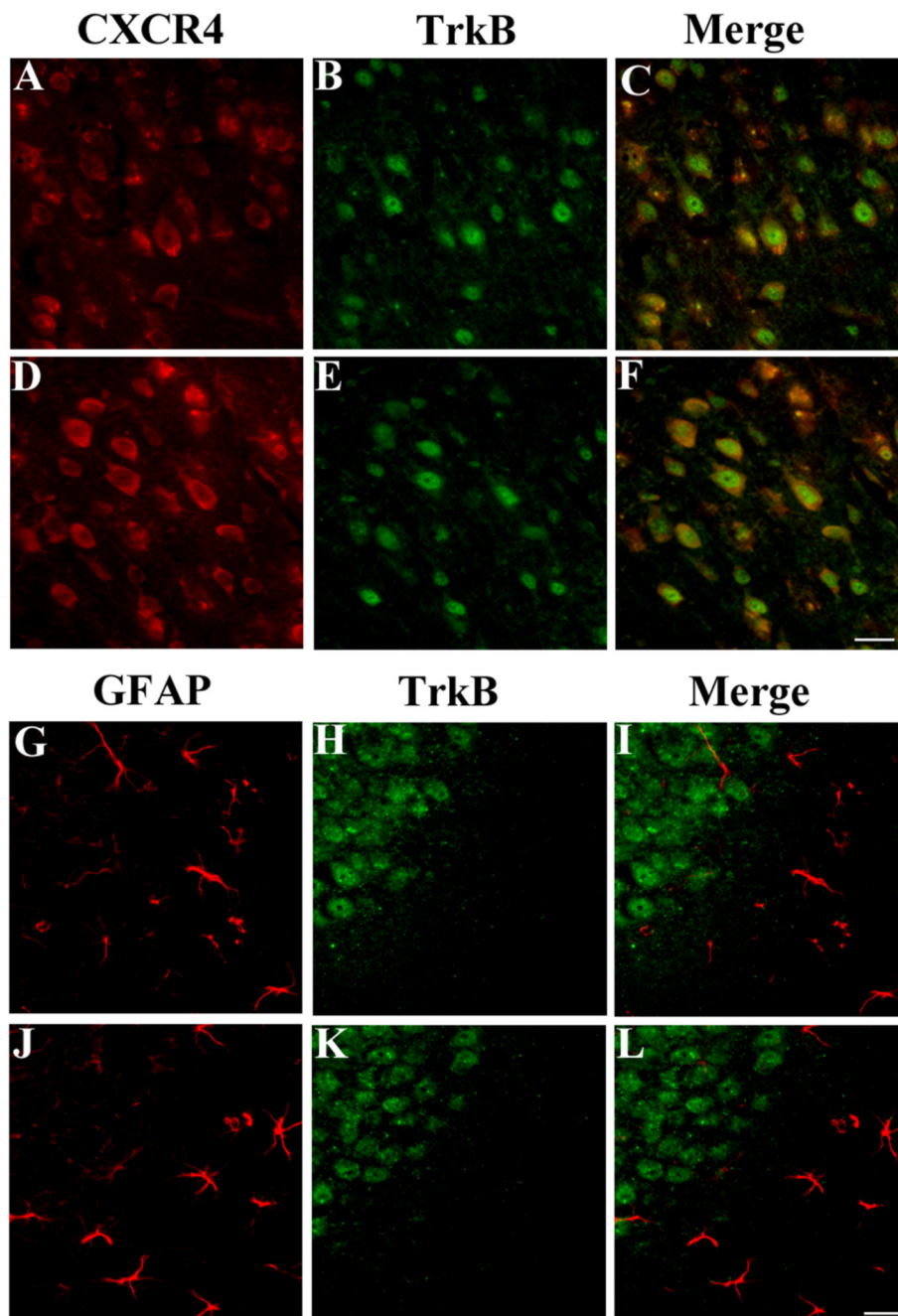
Figure 4. CXCR4 co-localizes with TrkB in neurons
Cortical sections from WT (A–C) and BDNF+/− mice (D–F) were stained with CXCR4 antibody (red) and an antibody that recognizes the extracellular domain of TrkB (αtrkB$_{23-36}$, green). In C and F, please note that a number of CXCR4 positive cells are also TrkB positive. Sections from the hippocampus of WT (G–I) and BDNF+/− mice (J–L) were stained for GFAP (red, G and J) and TrkB (green, H and K). No astrocytes were TrkB positive. Bar=50 µm.
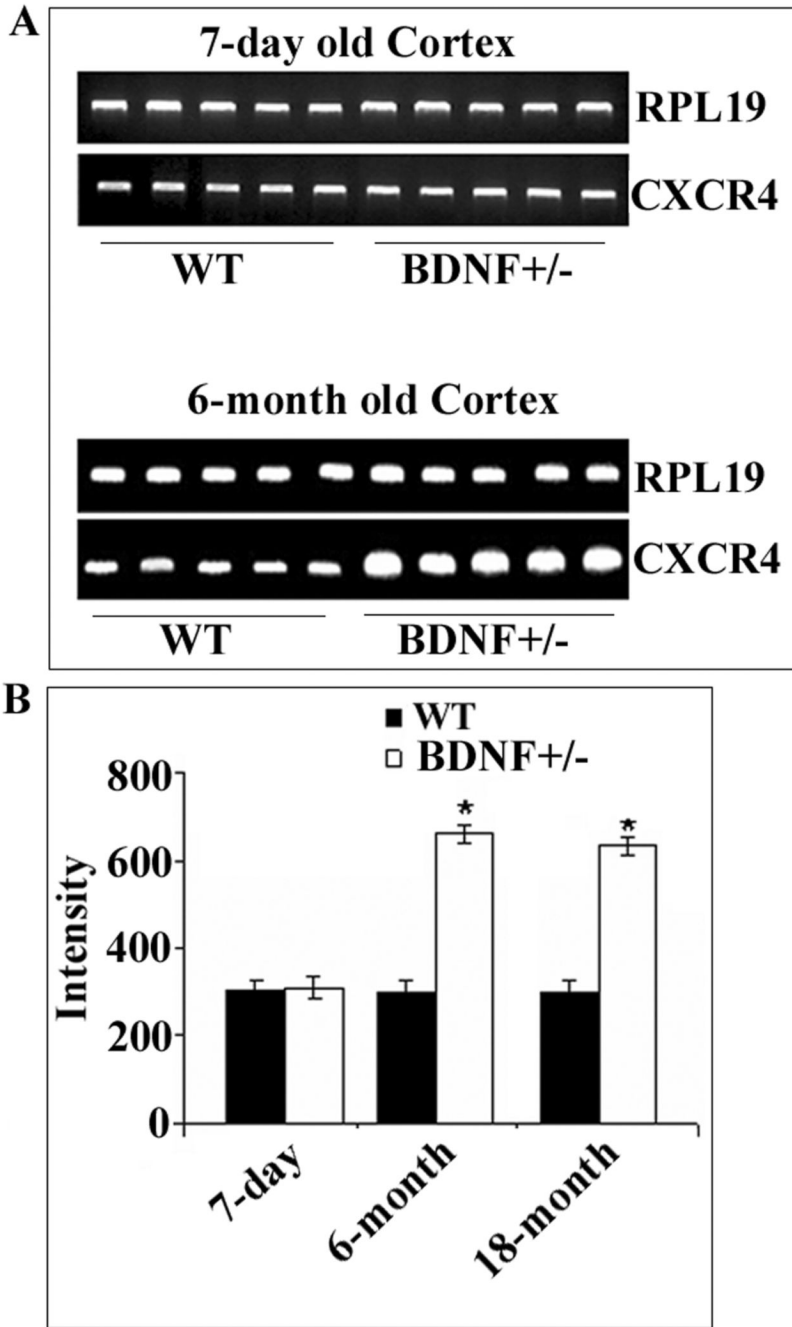
Figure 5. Age-dependent increase of CXCR4 in BDNF^{+/−} mice
Cortical CXCR4 mRNA levels were measured in 7-day-old pups, 6 and 18-month-old BDNF +/− mice and WT littermates. RNA extraction and RT-PCR was carried out as described in Experimental Procedures. **A**. Representative gels showing CXCR4 and RPL19 cDNAs from the cortex of 7-day old and 6-month old mice. **B**. Semi-quantitative analysis of CXCR4 mRNA was carried out by Quantity One 1-D analysis. Data, expressed as Intensity of cDNA bands, are the mean ± SEM of five independent samples. *p<.005 vs WT.
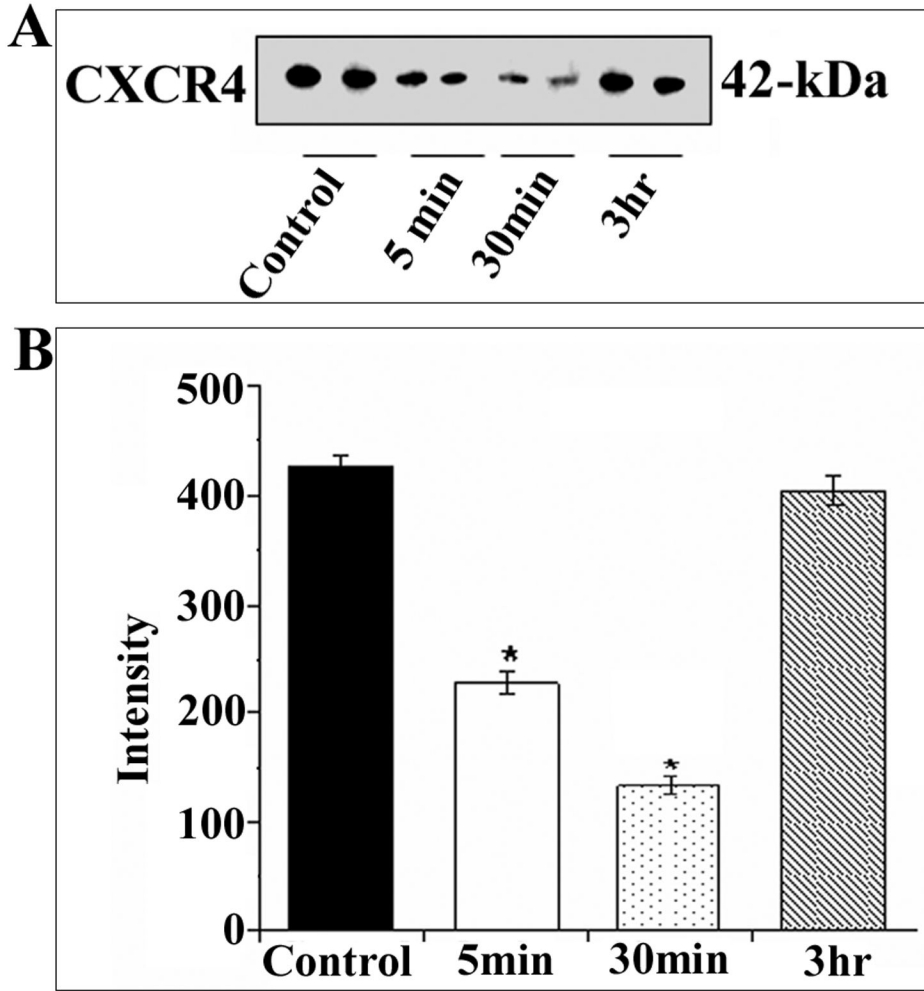
Figure 6. BDNF increases internalization of CXCR4
SY5YTB8 cells (Kim et al., 1999) were incubated with medium control (0.1% BSA) or medium containing BDNF (10 ng/ml) for 5 or 30 min or 3 hr. Cells were then washed and treated with 2 mM NHS-LC-biotin for 30 min at 4°C to biotinylate cell surface proteins. Biotinylated proteins were precipitated with immobilized streptavidin. Precipitates were then electrophoresed on SDS-polyacrylamide gels and subjected to CXCR4 immunoblotting. **A**. Example of immunoblot. **B**. Immunoreactive bands were scanned and quantified by Quantity One 1-D Analysis Software. Data, expressed as intensity of immunoreactive bands, are the mean ± SEM of three independent experiments. *p<0.05 vs control.
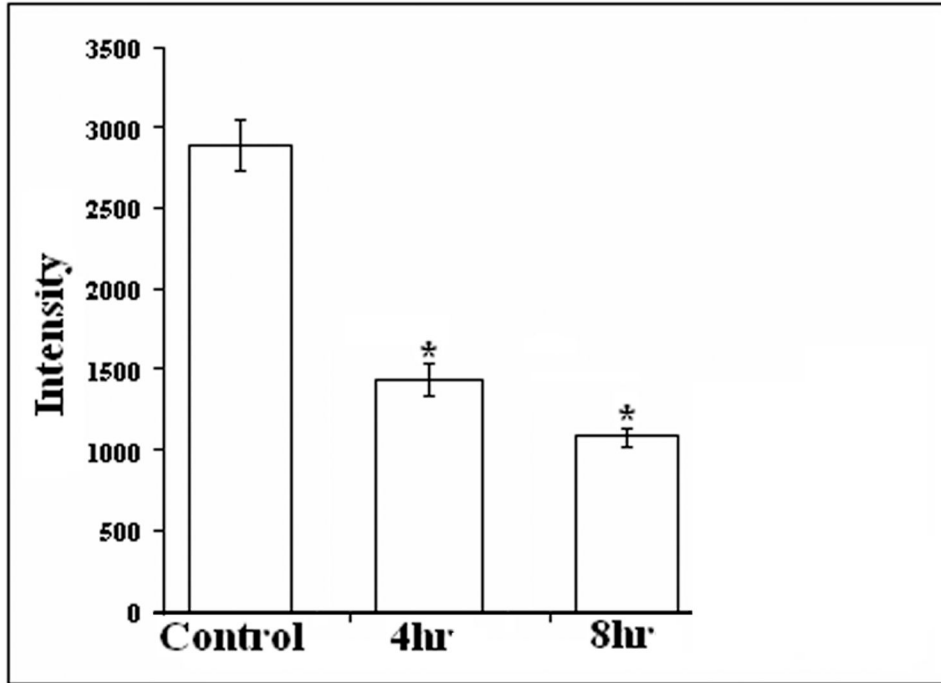
Figure 7. BDNF decreases CXCR4 mRNA levels
SY5YTB8 cells were exposed to medium control (0.1% BSA) or containing BDNF (10 ng/ml) for 4 and 8 hr and CXCR4 mRNA levels were measured by RT-PCR using primers designed to amplify human CXCR4 (see Table 1). Bands corresponding to CXCR4 cDNA were quantified using Quantity One 1-D Analysis Software. Data, expressed as intensity of bands, are the mean ± SEM of three independent experiments done (n=9). *p<0.05 vs control.
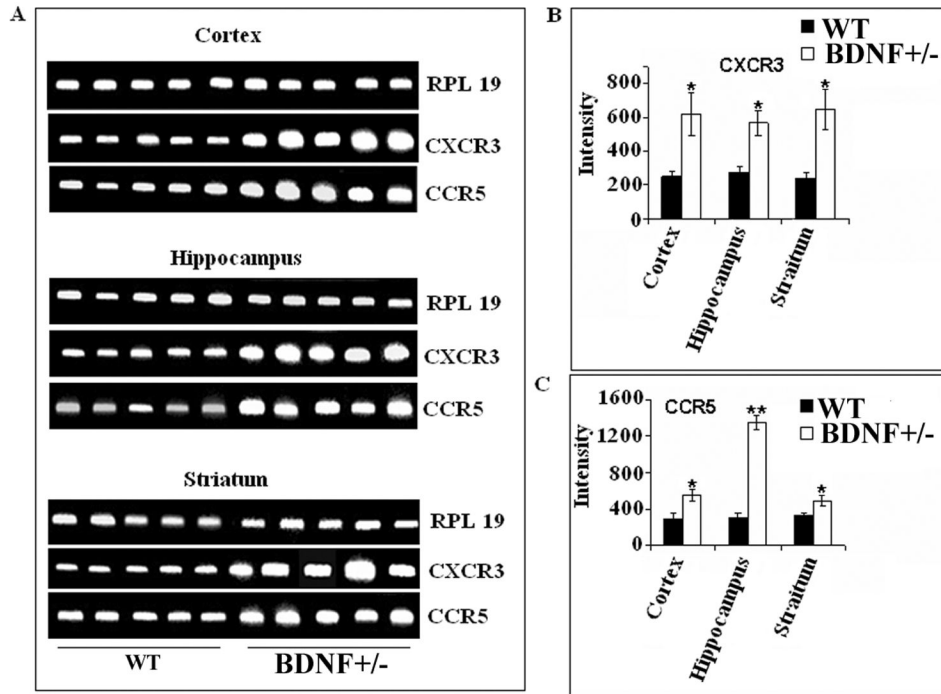
Figure 8. Modulation of chemokine receptors by BDNF is area-specific
Total RNA was extracted from the indicated brain areas of 6-month-old BDNF$^{+/-}$ mice or WT littermates. RT-PCR was performed using primers designed to amplify mouse CCR5 and CXCR3. **A**. Representative gels showing CCR5, CXCR3 and RPL19 cDNAs from the indicated brain areas. **B**. Semi-quantitative analysis of chemokine receptor cDNAs was carried out by Quantity One 1-D analysis. Data, expressed as intensity of cDNA bands, are the mean ± SEM of five independent samples. *p<0.05; **p<0.001 vs WT.

Table 1

Sense and antisense primers used for RT-PCR.

Chemokine	Species	gene bank number	Sequence
CXCR4	Human	AK129916	AGCGAGGTGGACATTCATCTGTT (1087–1109)
			CGTGATTCACTACACGCTCTGG (1418–1439)
Cyclophilin	Human	NM_021130	TCCTGCTTTCACAGAATTATTCC (234–256)
			ATTCGAGTTGTCCACAGTCAGC (558–579)
RPL 19	Mouse	BC010710	GGTACTGCCAATGCTCGGAT (264~273)
			TCCTTGGACAGAGTCTTGATGA (558~579)
CXCR4	Mouse	U59760	GGTCTGGAGACTATGACTCC (85~104)
			CACAGATGTACCTGTCATCC (589~609)
CXCR3	Mouse	AF045146	GAGGTTAGTGAACGTCAAGTG (9–30)
			GGGGTCCCTGCGGTAGATCTG (468–489)
CCR5	mouse	AF019772	TCAGTTCCGACCTATATCTATG (16–37)
			GTGGAAAATGAGGACTGCATGT (535–556)