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## **β-Funaltrexamine inhibits chemokine (CXCL10) expression in normal human astrocytes**

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### **Abstract**

Neuroinflammation is an integral component of neurodegenerative disorders, CNS infection and trauma. Astroglial chemokines, such as CXCL10, are instrumental in neuroinflammatory signaling as well as neurotoxicity. We have utilized proinflammatory-induced CXCL10 expression in normal human astrocytes (NHA) as a model in which to assess the anti-inflammatory actions of the selective, *mu*-opioid receptor (MOR) antagonist, β-funaltrexamine (β-FNA). Interferon (IFN)γ + HIV-1 Tat-induced CXCL10 expression (secreted protein and mRNA) was inhibited by co-treatment with β-FNA. Neither the MOR-selective antagonist, D-Phe-Cys-Tyr-D-Trp-Arg-Pen-Thr-NH<sub>2</sub> (CTAP) nor the nonselective opioid receptor antagonist, naltrexone inhibited IFNγ + HIV-1 Tat-induced CXCL10 expression. Furthermore, co-treatment with excess CTAP or naltrexone did not prevent β-FNA mediated inhibition of IFNγ + HIV-1 Tat-induced CXCL10 expression. Additionally, we utilized an inhibitor of NF-κB activation (SN50) to demonstrate that IFNγ + HIV-1 Tat-induced CXCL10 expression is NF-κB-dependent in NHA. Subsequent experiments revealed that β-FNA did not significantly affect NF-κB activation. Interestingly, we discovered that β-FNA inhibited p38 activation as indicated by decreased expression of phospho-p38. Together, these findings suggest that the inhibitory actions of β-FNA are MOR-independent and mediated, in part, via a transcriptional mechanism. These findings add to our understanding of the mechanism by which chemokine expression is inhibited by β-FNA. In conjunction with future investigations, these novel findings are expected to provide insights into the development of safe and effective treatments for neuroinflammation.

### **Keywords**

astroglial; HIV-1; *mu* opioid receptor; neuroinflammation; interferon γ

### **1. Introduction**

HIV-1 enters the central nervous system (CNS) within a matter of weeks following systemic infection (Gray et al., 1993) and can presumably persist for years (Kramer-Hammerle et al., 2005; Thompson et al., 2011). Approximately 50–60% of HIV-1 infected individuals suffer from HIV-associated neurocognitive disorders (HAND) (Giunta et al., 2006; Ozdener, 2005). The incidence of the most severe form of neurocognitive

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involvement, HIV-1 associated dementia (HAD), has been reduced with the advent of antiretroviral therapy; however, reports of more subtle forms of CNS impairment are increasing (Ances and Ellis, 2007; Cysique and Brew, 2009; Fischer-Smith and Rappaport, 2005). The exact mechanism by which HIV-1 causes these neuropathologies is not completely understood; however increasing evidence suggests neuronal damage results in part from microglial and astroglial derived cytokines and chemokines (Deshpande et al., 2005; Gonzalez-Scarano and Martin-Garcia, 2005; Minagar et al., 2002; Navia et al., 1986; Zhou et al., 2004).

The chemokine interferon  $\gamma$  inducible protein-10 (CXCL10) is among the proinflammatory molecules implicated in HIV-1 associated neuropathogenesis (Chang et al., 2004; Cinque et al., 2005; Eugenin et al., 2006; Lane et al., 2003; van Marle et al., 2004). CXCL10 is a member of the CXC or  $\alpha$ -chemokine family; all members of this family contain four highly conserved cysteine residues with the first two separated by a single amino acid (Bajetto et al., 2002; Luster et al., 1985). CXCL10 is also subclassified as ELR-negative due to the lack of a conserved glutamate-leucine-arginine (ELR) motif at the N-terminus. CXCL10 is a small secreted protein, instrumental in physiological and pathological processes, but most fully characterized as a chemoattractant for activated T cells (Taub et al., 1993), monocytes/macrophages (Taub et al., 1993) and microglia (Flynn et al., 2003). In addition to recruiting inflammatory cells, CXCL10 induces astroglial proliferation (Flynn et al., 2003) and is directly neurotoxic (Sui et al., 2004; Sui et al., 2006). Substantial evidence has been documented in terms of a role for CXCL10 in HIV-1 neuropathogenesis. For instance, CXCL10 is elevated in the CNS of patients with HIV-1 associated dementia (HAD) and HIV-1 encephalitis (Conant et al., 1998; Kelder et al., 1998; Kolb et al., 1999). Also, CXCL10 levels in the CSF of HIV-1 infected individuals are positively correlated with neurological deficits (Kolb et al., 1999). In autopsy brain tissue, CXCL10 protein expression is greater in HIV<sup>+</sup> individuals compared to HIV<sup>-</sup> subjects and is detected primarily in astrocytes located in proximity to microglial nodules and activated microglia (Sanders et al., 1998). Furthermore, CXCL10 stimulates HIV-1 replication in macrophage and peripheral blood lymphocytes (Lane et al., 2003).

Viral proteins released from HIV-1 infected cells are also involved in the pathogenesis of HAD (Ensoli et al., 1993; Ghafouri et al., 2006; Westendorp et al., 1995). For example, the virally encoded transactivator protein, Tat, is directly neurotoxic (King et al., 2006), but also enters uninfected astrocytes and microglia and induces the expression of numerous proinflammatory molecules (Nath, 2002). HIV-1 Tat induces the expression of chemokines, including CXCL10, in astrocytes (Khiati et al., 2010; Kutsch et al., 2000). Furthermore, HIV-1 Tat-induced CXCL10 in human astrocytes is potentiated by cytokines, including TNF $\alpha$  and IFN $\gamma$  (Williams et al., 2009).

While antiretroviral therapy (ART) inhibits HIV-1 replication in the periphery, few of these therapeutic agents readily enter the CNS or reduce neuroinflammatory responses associated with HIV-1 (Chang et al., 2003; Chang et al., 2004; Nath and Sacktor, 2006). In fact, there is ongoing neuroinflammation in ART treated patients (Anthony et al., 2005). Therefore, there has been considerable interest in identifying agents which attenuate neuroinflammatory responses activated by HIV-1 infection (Agrawal et al., 2007; Chen et al., 2003; Dou et al., 2005; Wilson et al., 2006). For example, treatment of SIV-infected macaques with the antibiotic minocycline reduced severity of encephalitis and decreased expression of several neuroinflammatory molecules including chemokines (Zink et al., 2005). Chemokines, such as CXCL10, are potentially key molecules which could be targeted as a means of reducing HIV-1 associated neuropathogenesis (Ansari et al., 2006; Biber et al., 2006; Eugenin et al., 2006; Lane et al., 2003). While not specifically targeted to chemokines, another intriguing strategy that has been employed to attenuate inflammation-mediated neuropathogenesis is

treatment with naloxone (Liao et al., 2003; Liu et al., 2000; Liu et al., 2002; Liu and Hong, 2003). Naloxone is well characterized as a non-selective opioid receptor antagonist; however, naloxone reduces neuroinflammation via mechanisms that do not require binding to opioid receptors. Such opioid receptor independent actions of naloxone include prevention of bacterial lipopolysaccharide (LPS)-binding to microglia (Liu et al., 2000) and reduced microglial superoxide production (Liu et al., 2002). Furthermore, we more recently determined that TNF $\alpha$  induced CXCL10 protein expression in human astroglial cells is dose-dependently inhibited by the selective, *mu*-opioid receptor (MOR) antagonist,  $\beta$ -funaltrexamine ( $\beta$ -FNA). These findings were particularly interesting given that  $\beta$ -FNA inhibited TNF $\alpha$ -induced CXCL10 expression in part through a MOR-independent mechanism (Davis et al., 2007). Given that  $\beta$ -FNA is centrally penetrating (Labuz et al., 2007), it holds promise as a therapeutic agent for select neuropathologies.

The mechanism of action of  $\beta$ -FNA in both behavioral (Ward et al., 1982) and *in vitro* assays (Liu-Chen et al., 1990) is initially reversible *kappa*-opioid receptor (KOR) agonism followed by irreversible MOR antagonism. However, our studies suggest that it is not the interaction of  $\beta$ -FNA with classical opioid receptors that causes the inhibition of proinflammatory-induced CXCL10 expression (Davis et al., 2007). Prior to the present report, our studies have focused on  $\beta$ -FNA effects on inflammatory signaling in the human A172 astroglial cell line (Davis et al., 2007; 2008). However, we have now expanded our studies and have determined that  $\beta$ -FNA also has anti-inflammatory actions in normal human astrocytes (NHA). Furthermore, the data support our previous findings that the anti-inflammatory actions of  $\beta$ -FNA not mediated through the MOR.

## 2. Materials and methods

### 2.1. Cell culture

Normal human astrocytes (NHA, cat# HA1800; ScienCell Research Laboratories, Carlsbad, CA) were cultured using reagents and procedures provided by the supplier. Cultures were maintained in Astrocyte Medium (cat.# 1801) containing 2% fetal bovine serum, growth supplement (cat.# 1852), and penicillin/streptomycin solution (cat.# 0503). All cultures were maintained in a humidified incubator at 37°C, 5% CO<sub>2</sub> and 95% air. Experimental cultures (passages 1–3) were seeded at a cell density ( $1 \times 10^4/\text{cm}^2$ ) that provided 80–90% confluence at the time of treatment. Culture medium was replenished every 48 h unless otherwise noted.

### 2.2. Proinflammatory stimulation

In order to induce CXCL10 expression and nuclear translocation of NF- $\kappa$ B, growth medium in astroglial cultures was replaced with serum free medium containing human recombinant IFN $\gamma$  (10 ng/ml; Peprotech, Rocky Hill, NJ) and HIV-1 Tat<sub>1–72</sub> (100 nM; Prepared by Phil Ray, University of Kentucky Neuroscience, Department of Neurology). The duration of stimulation was 24 h for induction of CXCL10 protein expression, 8 h for CXCL10 mRNA expression and 10–270 min for NF- $\kappa$ B activation.

### 2.3. Opioid compounds

The MOR-selective antagonists included  $\beta$ -FNA (10  $\mu$ M; obtained from the NIDA reagent supply program) and D-Phe-Cys-Tyr-D-Trp-Arg-Pen-Thr-NH<sub>2</sub> (CTAP; 30, 100  $\mu$ M; Sigma, St. Louis, MO). Naltrexone (30, 100  $\mu$ M; Sigma) was used as a nonselective opioid receptor antagonist. In all experiments, opioid reagents were added to cell cultures at the time of proinflammatory stimulation.

#### 2.4. CXCL10 protein expression

A standard dual-antibody solid phase immunoassay (ELISA Development Kit; Peprotech, Rocky Hill, NJ) was used for quantitation of secreted CXCL10 in cell culture supernatants, according to the manufacturer's instructions and as previously described (Davis et al., 2007).

#### 2.5. CXCL10 mRNA expression

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) and CXCL10 mRNA assessed by real-time PCR using the SYBR Green detection method using the basic protocol previously described (Davis et al., 2008). The RT-PCR primer pair sets were obtained from Invitrogen and amplification from first strand cDNA was performed as instructed by the supplier. The primer sequences for CXCL10 were AACCTCCAGTCTCAGCACCATGAA (forward) and AGGTACAGCGTAAGGTTCTAGAGAG (reverse); the primer pairs for GAPDH were GAGTCAACGGATTTGGTCGT (forward) and TTGATTTTGGAGGGATCTCG (reverse). Relative quantification of gene expression was evaluated using the comparative cycle threshold ( $C_T$ ) method (Hettinger et al., 2001) as previously described (Davis et al., 2008). Expression of CXCL10 mRNA was normalized to the expression of the endogenous control, GAPDH mRNA. Relative expression was calculated using the comparative  $\Delta \Delta C_T$  method and fold change calculated using  $2^{-\Delta\Delta C_T}$ .

#### 2.6. Inhibitor of NF- $\kappa$ B activation

To assess the role of NF- $\kappa$ B in IFN- $\gamma$  + Tat-induced CXCL10 expression, NHA were stimulated in the presence of SN50 (Biomol; Farmingdale, NY), a cell permeable inhibitory peptide containing the nuclear localization sequence of NF- $\kappa$ B p50, which prevents nuclear translocation of the active NF- $\kappa$ B complex (Lin et al., 1995). As done previously, (Pahan et al., 2001; Yang et al., 2005), cells were pre-incubated with 50  $\mu$ M SN50 or 50  $\mu$ M of the inactive mutant peptide SN50M, for 1 h prior to stimulation.

#### 2.7. Nuclear translocation of NF- $\kappa$ B p65

NF- $\kappa$ B activation, as determined by increased levels of the active (DNA-binding) form of NF- $\kappa$ B p65 in the nucleus, was assessed using the p65 Transcription Factor kits (Thermo Scientific, Rockford, IL) as we previously described (Tousi et al., 2010). Nuclear protein was obtained according to the protocol described in our previous report (Davis and Syapin, 2004) and luminescence of the labeled NF- $\kappa$ B-DNA product was then measured.

#### 2.8. p38 activation

Western blotting was done as previously described (Das et al., 2011) to determine phospho-p38 levels, as a measure of p38 activation. Following experimental treatments, cells were washed twice with PBS and cytosolic extracts prepared using Cytoplasmic Extraction Reagent (Thermo Scientific, Rockford, IL). Briefly, fifty micrograms of total protein were loaded on 10% SDS-PAGE gel, electrophoresed and then transferred to PVDF membrane. Antibodies used were anti-Phospho-p38 and anti-p38 (both from Cell Signaling, Danvers, MA) and were used in the dilution range of 1:1000. The blots were scanned in a phosphoimager Typhoon 9410 (GE Healthcare, Uppsala, Sweden) using enhanced chemifluorescence reagent (GE Healthcare, UK). Densitometric analysis of western blot protein bands was done using Image J (National Institute of Health).

#### 2.9. Determination of total protein content

Total cellular/nuclear protein levels were determined using the bicinchoninic acid (BCA) protein assay as previously described (Davis et al., 2002) in order to normalize data when appropriate.

## 2.10. Statistical analysis

Prism™ version 4.0 software (GraphPad Inc., San Diego, CA) was used for figure presentation and statistical analysis. Analyses included one-way analysis of variance (ANOVA) with Neuman-Kuels multiple comparisons or a Dunnett's comparison. Additionally, a two-ANOVA was used, followed by Bonferroni's multiple comparisons. Data are presented as mean + S.E.M and a probability (p) of < 0.05 was accepted as demonstrating statistically significant differences between groups. The *n* values and number of independent experiments from which the data were obtained are provided in the individual figure legends.

## 3. Results

### 3.1. Effects of $\beta$ -FNA on IFN $\gamma$ and Tat-induced CXCL10 expression

Initially we assessed the induction of CXCL10 expression following stimulation with IFN $\gamma$  and Tat alone, and in combination. Next we determined the effect of  $\beta$ -FNA on induced CXCL10 expression. Constitutive expression of CXCL10 protein in NHA was negligible; whereas, 24 h exposure to either IFN $\gamma$  or Tat alone induced low levels of CXCL10 protein ( $192 \pm 73$  and  $136 \pm 49$  pg/ml, respectively; Fig. 1A). Concurrent exposure to IFN $\gamma$  and Tat resulted in a synergistic increase in CXCL10 protein expression ( $2711 \pm 411$  pg/ml; Fig. 1A). Given the robust induction of CXCL10 protein expression by IFN $\gamma$  + Tat, subsequent experiments used only this proinflammatory combination for stimulation of NHA. Co-exposure to  $\beta$ -FNA significantly inhibited IFN $\gamma$  + Tat-induced CXCL10 protein expression.

Consistent with CXCL10 protein expression data, constitutive CXCL10 mRNA levels were negligible in NHA, whereas, exposure to IFN $\gamma$  + Tat resulted in approximately a 1000-fold increase in CXCL10 mRNA expression 8 h post-stimulation (Fig. 1B). However, co-exposure to  $\beta$ -FNA significantly inhibited IFN $\gamma$  + Tat-induced CXCL10 mRNA expression.

### 3.2. Comparison of the effects of select MOR antagonists on IFN $\gamma$ + Tat-induced CXCL10 protein expression

Firstly, this series of experiments was performed to determine whether the anti-inflammatory actions are similar among different MOR antagonists. Secondly, we aimed to determine whether MOR signaling was involved in the anti-inflammatory actions of  $\beta$ -FNA. Thus, we were interested to know whether the effects of  $\beta$ -FNA could be antagonized by co-exposure to a second opioid receptor antagonist (in relative excess). Similar to our previous experiments, 10  $\mu$ M  $\beta$ -FNA significantly inhibited IFN $\gamma$  + Tat-induced CXCL10 protein expression (Fig. 2). Conversely, exposure to considerably higher concentrations (30 and 100  $\mu$ M) of either naltrexone (nonselective opioid receptor antagonist) or CTAP (MOR-selective antagonist) did not significantly affect IFN $\gamma$  + Tat-induced CXCL10 protein expression. Co-treatment with naltrexone (30 or 100  $\mu$ M) did not block the inhibition of CXCL10 protein expression caused by  $\beta$ -FNA (Fig. 2A). Likewise, co-treatment with CTAP (30 or 100  $\mu$ M) did not block the inhibition of CXCL10 protein expression caused by  $\beta$ -FNA (Fig. 2B).

### 3.3. Role of NF- $\kappa$ B in IFN $\gamma$ + Tat -induced CXCL10 protein expression

To confirm that IFN $\gamma$  + Tat induces CXCL10 expression in NHA via an NF- $\kappa$ B-dependent mechanism, we utilized SN50, a well characterized inhibitor of NF- $\kappa$ B nuclear translocation. Pretreatment of NHA with SN50 significantly inhibited IFN $\gamma$  + Tat-induced CXCL10 protein expression (Fig. 3). Conversely, pretreatment of NHA with the mutated form of this inhibitory molecule, SN50 Mut, had no effect on IFN $\gamma$  + Tat-induced CXCL10 protein expression (Fig. 3).

### 3.4. Effect of $\beta$ -FNA on IFN $\gamma$ + Tat -induced of NF- $\kappa$ B activation

This experiment was performed to determine whether  $\beta$ -FNA inhibits NF- $\kappa$ B activation, a process that is necessary for IFN $\gamma$  + Tat-induced CXCL10 protein expression. IFN $\gamma$  + Tat induced a time-dependent increase in NF- $\kappa$ B activation (Fig. 4). IFN $\gamma$  + Tat-induced NF- $\kappa$ B activation steadily increased over the first 90 min of stimulation, and then returned toward baseline by 270 min (Fig. 4). Two-ANOVA ( $\beta$ -FNA treatment  $\times$  time) followed by Bonferroni's multiple comparisons indicated that NF- $\kappa$ B activity was significantly elevated at 90 min ( $p < 0.05$  vs. 10 min). Two-ANOVA also indicated a significant effect of time ( $p < 0.004$ ) on IFN $\gamma$  + Tat-induced NF- $\kappa$ B activation; however,  $\beta$ -FNA did not significantly affect NF- $\kappa$ B activation ( $p = 0.59$ ) nor was an interaction detected ( $p = 0.62$ ) (Fig. 4).

### 3.5. Effect of $\beta$ -FNA on IFN $\gamma$ + Tat -induced of p38 activation

Activation of p38 is instrumental in the induction of CXCL10 expression, therefore, we were interested in determining whether this pathway is disrupted by  $\beta$ -FNA. Exposure to IFN $\gamma$  + Tat induced p38 activation as indicated by significantly increased phosphorylation of p38 following 10 min of stimulation (Fig. 5). Co-exposure to  $\beta$ -FNA significantly inhibited proinflammatory induced p38 activation as indicated by lower levels of phosphorylated p38 at 10 and 30 min post-stimulation.

## 4. Discussion

Neuroinflammation is associated with neurodegenerative disorders as well as CNS infection and trauma (Hirsch and Hunot, 2009; Kadiu et al., 2005; Persidsky and Gendelman, 2003; Poluektova et al., 2005; Vlodaysky et al., 2006). Chemokines are among the inflammatory mediators which are instrumental in response to CNS insults and the expression levels are frequently elevated with neuropathology (Cinque et al., 2005; Gonzalez et al., 2003; Kolb et al., 1999; Xia et al., 2000). The relative contribution of these chemokines to neuroprotection or neurotoxicity depends largely on the temporal and cellular context; in fact there is still much to learn about the specific involvement of individual chemokines in the various types of neuropathology. The chemokine CXCL10 can be directly neurotoxic and the involvement of this chemokine in HIV-1 associated neuropathogenesis has been well documented (Kolb et al., 1999; Sui et al., 2004; Sui et al., 2006).

Neutralization of CXCL10 as a means of reducing neuroinflammatory damage is of interest to our group as well as others (Davis et al., 2007; Glaser et al., 2004; Sorensen, 2004). We are particularly interested in the anti-inflammatory actions of  $\beta$ -FNA (Davis et al., 2007; 2008). We previously reported that  $\beta$ -FNA inhibits TNF $\alpha$ -induced CXCL10 expression in the human A172 astroglial cell line (Davis et al., 2007). We have expanded upon these previous studies and used primary astrocytes and proinflammatory stimuli germane to HIV-1 infection, IFN $\gamma$  and Tat, in order to study the anti-inflammatory effects of  $\beta$ -FNA. Exposure to IFN $\gamma$  + Tat synergistically enhanced CXCL10 expression in NHA. However, co-exposure to  $\beta$ -FNA inhibited IFN $\gamma$  + Tat-induced induced CXCL10 expression (protein and mRNA), which suggests that the inhibitory actions of  $\beta$ -FNA are, in part, via a transcriptional mechanism. Together with our previous report (Davis et al., 2007), these findings suggest that inhibition of proinflammatory-induced CXCL10 expression by  $\beta$ -FNA is not unique to human A172 astroglial cells, as inhibition also occurs in NHA.

Astrocytes can express multiple opioid receptor types and these receptors are differentially expressed among brain regions (Ruzicka et al., 1995; Stiene-Martin et al., 1998). With regards to its mechanism of action,  $\beta$ -FNA exhibits irreversible antagonism at MOR (Ward et al., 1982). We were the first to report the anti-inflammatory actions of  $\beta$ -FNA in human astroglial cells (Davis et al., 2007; 2008), and the data indicated a MOR-independent mechanism of action. For instance, we found that the relatively potent MOR agonist,

fentanyl, dose-dependently inhibited TNF $\alpha$ -induced CXCL10 expression in A172 cells. However, neither the non-selective MOR antagonist, naltrexone, nor the highly selective MOR antagonist,  $\beta$ -FNA, blocked the fentanyl-mediated inhibition of CXCL10 expression (Davis et al., 2007). In fact,  $\beta$ -FNA was a more potent inhibitor of CXCL10 expression in A172 cells than was fentanyl (Davis et al., 2007). Subsequently, we performed a series of experiments to further address the potential involvement of opioid receptors, including MOR, KOR and nociceptin/orphanin FQ receptor (ORL). We found that  $\beta$ -FNA actions are not modulated by another MOR selective antagonist, CTAP (at a concentration 3-fold higher than  $\beta$ -FNA), further suggesting that inhibition of CXCL10 expression by  $\beta$ -FNA is not through actions at the MOR (unpublished data). Similarly, KOR and ORL do not appear to be involved in  $\beta$ -FNA effects on CXCL10 expression as nor-binaltorphimine and [Nphe<sup>1</sup>]-nociceptin<sub>1-13</sub> amide, respectively (at concentrations 3-fold higher than  $\beta$ -FNA), failed to block  $\beta$ -FNA effects (unpublished data).

Rather unexpected effects of  $\beta$ -FNA on chemokine (IL-8 and MIP-1 $\beta$ ) expression in human astrocytes have been reported by others (Mahajan et al., 2002). In addition to blocking morphine-mediated suppression of constitutive chemokine expression,  $\beta$ -FNA alone up-regulated chemokine expression in human astrocytes (Mahajan et al., 2002). These investigators suggested that  $\beta$ -FNA may be activating at another type of opioid family receptor such as ORL or another as yet unidentified site. Others have reported that naloxone has anti-inflammatory and neuroprotective actions that are unrelated to MOR antagonism (Dutta et al., 2008; Liu et al., 2000; Liu et al., 2002; Zhang et al., 2010). For instance, LPS-stimulated microglial-induced neuronal toxicity is blocked by naloxone, in part, by blocking LPS binding to microglia. These neuroprotective actions were mediated by both the inactive and active stereoisomers (with respect to MOR antagonism), (+) naloxone and (-)-naloxone, respectively (Liu et al., 2000). This same group later demonstrated that A $\beta$ -induced neurotoxicity is reduced by naloxone, reportedly by inhibiting microglial superoxide production in a MOR-independent manner (Liu et al., 2002). More recently, it was reported that in neuron-glia cultures, MnCl<sub>2</sub> + LPS-induced dopaminergic toxicity was prevented by pre-treatment with naloxone (Zhang et al., 2010). Furthermore, cytokine release and generation of reactive oxygen species by microglia and astrocytes was reduced by naloxone pre-treatment (Zhang et al., 2010).

In the present study, again we used co-exposure to excess (3- and 10-fold relative to  $\beta$ -FNA) opioid receptor antagonists as a means of assessing the potential involvement of opioid receptors in  $\beta$ -FNA mediated inhibition of CXCL10 in NHA. The fact that neither a non-selective opioid receptor antagonist (naltrexone) nor a MOR-selective antagonist (CTAP) blocked  $\beta$ -FNA inhibition of IFN $\gamma$  + Tat-induced CXCL10 expression, further supports a MOR-independent mechanism of action in human astrocytes. It is also important to note that neither naltrexone nor CTAP alone inhibit IFN $\gamma$  + Tat-induced CXCL10 expression, further highlighting the novel anti-inflammatory actions of  $\beta$ -FNA. Interestingly, there was a trend for CTAP alone to increase IFN $\gamma$  + Tat-induced CXCL10 expression. Thus, the slight attenuation of  $\beta$ -FNA-inhibition of IFN $\gamma$  + Tat-induced CXCL10 expression by 100  $\mu$ M CTAP likely reflects the stimulatory action of CTAP rather than antagonism at MOR.

Given the apparent MOR-independent nature of the anti-inflammatory actions of  $\beta$ -FNA, we investigated another potential intracellular target important in CXCL10 induction, NF- $\kappa$ B. While proinflammatory induction of CXCL10 has been shown to be NF- $\kappa$ B-dependent we needed to confirm this in our experimental model. Indeed, utilizing the inhibitor of NF- $\kappa$ B nuclear translocation, SN50, we demonstrated that IFN $\gamma$  + Tat-induced CXCL10 expression is NF- $\kappa$ B-dependent in NHA. NF- $\kappa$ B activation, as determined by levels of NF- $\kappa$ B p65-DNA binding activity, was not inhibited by  $\beta$ -FNA. Likewise, NF- $\kappa$ B p50-DNA binding activity was not significantly affected by  $\beta$ -FNA (data not shown). These data suggest that

$\beta$ -FNA inhibition of CXCL10 expression is not through an NF- $\kappa$ B p65 mediated mechanism. These findings are consistent with our observations in A172 cells where  $\beta$ -FNA did not significantly affect nuclear levels NF- $\kappa$ B p65 (as determined by immunoblot) (Davis et al., 2007). Additionally, it may be that  $\beta$ -FNA enhances the degradation or nuclear export of NF- $\kappa$ B proteins. However, before a role for NF- $\kappa$ B signaling is completely abandoned as a target of  $\beta$ -FNA, future investigations should assess other proteins in the NF- $\kappa$ B pathway, as well as more functional measures including dimerization of the various NF- $\kappa$ B subunits and transcriptional activation. For instance, we have found that TNF $\alpha$ -induced NF- $\kappa$ B driven promoter activity in THP-1 monocytes is inhibited by  $\beta$ -FNA (unpublished data). These findings also hint at the possibility that  $\beta$ -FNA acts at multiple sites, resulting in stimulus-dependent effects.

Given the importance of p38 activation in inflammatory signaling in glia, including CXCL10 expression (Williams et al., 2009), we also investigated the effects of  $\beta$ -FNA on this signaling molecule. Importantly, we discovered that  $\beta$ -FNA inhibits IFN $\gamma$  + Tat-induced p38 activation in human astroglial cells. This finding provides insight into the mechanism underlying the anti-inflammatory actions of  $\beta$ -FNA. It remains to be determined whether  $\beta$ -FNA alters phosphorylation per se or targets upstream signaling events. Ongoing and future investigations are expected to provide new details about the mechanism of action for the anti-inflammatory actions of  $\beta$ -FNA. However, we have demonstrated that the inhibitory actions of  $\beta$ -FNA on CXCL10 expression are MOR-independent and, in part, are transcriptional.

CXCL10 is not the only inflammatory molecule affected by  $\beta$ -FNA. For instance, we previously reported that expression of inducible nitric oxide synthase is transcriptionally inhibited by  $\beta$ -FNA (Davis et al., 2008). Interestingly, the expression of other inflammatory molecules such as CXCL10/IL-8 and CCL2/MCP-1, are much less sensitive to the inhibitory actions of  $\beta$ -FNA (unpublished data). Further investigation of  $\beta$ -FNA effects on CXCL8, CCL2, and other inflammatory mediators are therefore warranted.

The anti-inflammatory actions of  $\beta$ -FNA identified support and add to the findings of others (Liu et al., 2000; Liu and Hong, 2003) which have indicated MOR-independent, anti-inflammatory actions of naloxone (a non-specific, opioid receptor antagonist). These investigators found that lipopolysaccharide (LPS)-stimulated microglia mediated pronounced neuronal toxicity when added to rat mesencephalic mixed neuron-glia cultures (Liu et al., 2000). Naloxone reportedly provided neuroprotection by blocking LPS binding to microglia (Liu et al., 2000). Furthermore, it was determined that the inactive stereoisomer, (+)-naloxone, was equally effective as (-)-naloxone (Liu et al., 2000). These investigators also determined that A $\beta$  (1–42)-induced neurotoxicity was inhibited by naloxone in an opioid receptor-independent mechanism, reduced microglial superoxide production (Liu et al., 2002). Furthermore, additional information about opioid actions that are not mediated through classical opioid receptors has been recently reviewed (Hutchinson et al., 2011). For instance, LPS-induced toll-like receptor-4 (TLR4) activation is inhibited by (+)-naloxone as well as (+)-naltrexone (Hutchinson et al., 2008).

Thus, there is ongoing interest in the MOR-independent actions of opioid compounds. The findings presented herein add to this knowledge base and highlight the potential for  $\beta$ -FNA as adjunct therapy for neuropathologies involving neuroinflammation. However, further investigation is warranted in order to more fully define the mechanism of action for the anti-inflammatory actions of  $\beta$ -FNA.



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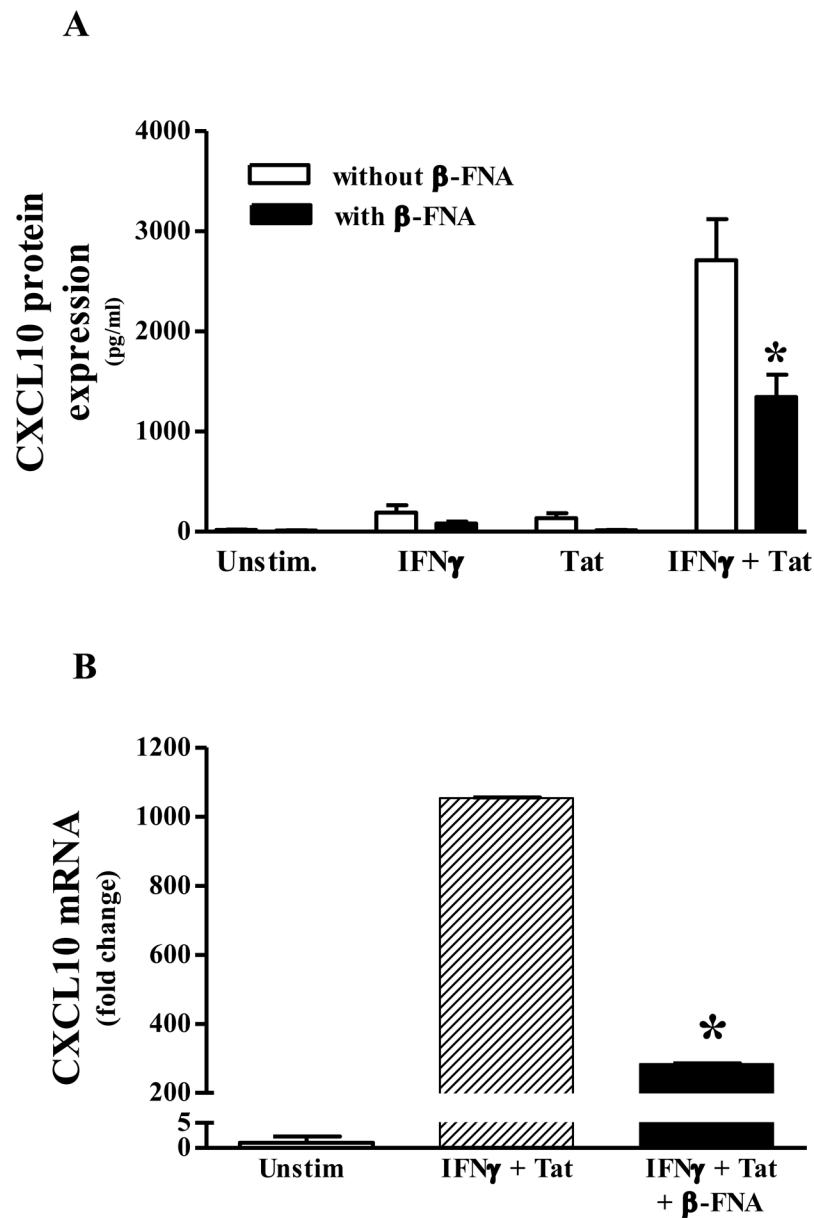
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### Highlights

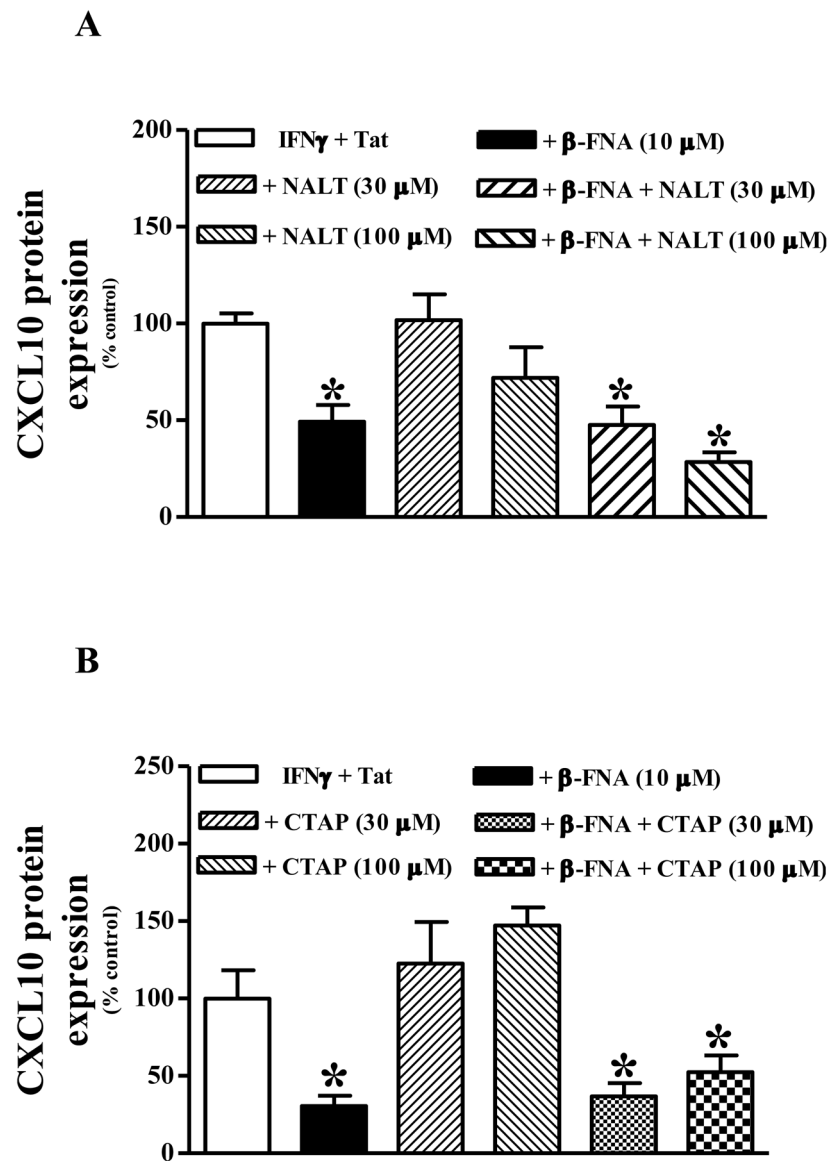
- IFN $\gamma$  + HIV-1 Tat induces CXCL10 expression in human astrocytes
- $\beta$ -FNA inhibits IFN $\gamma$  + HIV-1 Tat induced CXCL10 expression
- inhibitory actions of  $\beta$ -FNA are not mediated through the *mu*-opioid receptor
- $\beta$ -FNA does not inhibit IFN $\gamma$  + HIV-1 Tat induced NF- $\kappa$ B activation
- $\beta$ -FNA inhibits IFN $\gamma$  + HIV-1 Tat induced p38 activation



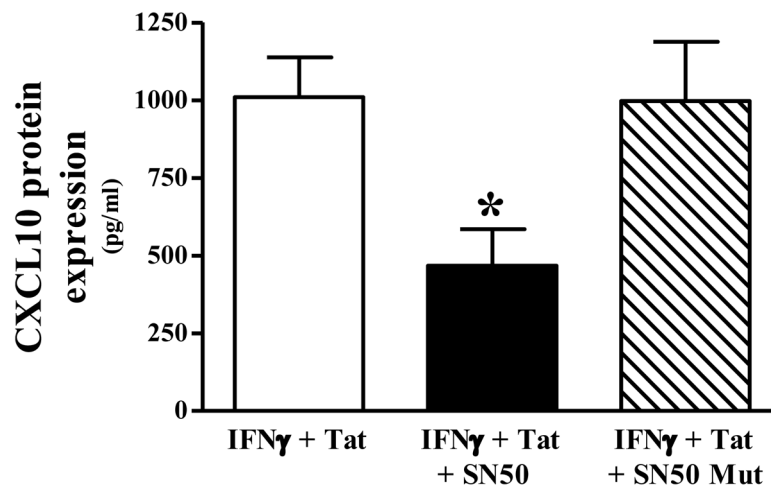
**Fig. 1.**  $\beta$ -FNA inhibits proinflammatory-induced CXCL10 expression in normal human astrocytes. **Panel A**)  $\beta$ -FNA effects on CXCL10 protein expression: In the presence or absence of  $\beta$ -FNA (10  $\mu$ M), normal human astrocytes were exposed to human recombinant IFN $\gamma$  (10 ng/ml), HIV-1 Tat<sub>1-72</sub> (100 nM), IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>, or serum free medium alone for 24 h. CXCL10 protein levels in the media were then measured by ELISA. The data represent mean + S.E.M of 9 independent experiments. Two-way ANOVA ( $\beta$ -FNA treatment  $\times$  stimulus) indicated a significant effect of  $\beta$ -FNA ( $p < 0.009$ ), stimulus ( $p < 0.0001$ ) and an interaction ( $p < 0.0005$ ). Bonferroni's multiple comparisons indicated that  $\beta$ -FNA significantly (\*  $p < 0.001$ ) inhibited IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>-induced CXCL10 expression. **Panel B**)  $\beta$ -FNA effects on CXCL10 mRNA expression: In the presence or absence of  $\beta$ -FNA (10  $\mu$ M), normal human astrocytes were exposed to human recombinant IFN $\gamma$  (10 ng/ml) + HIV-1 Tat<sub>1-72</sub> (100 nM) or in serum free medium alone for 8 h. Total RNA was

isolated and CXCL10 and GAPDH mRNA assessed by real-time PCR. The data represent mean + S.E.M of 6 independent experiments. Analysis of  $\Delta C_T$  values by one-way ANOVA followed by Newman-Keuls multiple comparisons indicated that  $\beta$ -FNA significantly inhibited IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>-induced CXCL10 mRNA expression. \*  $p < 0.05$  vs. IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>

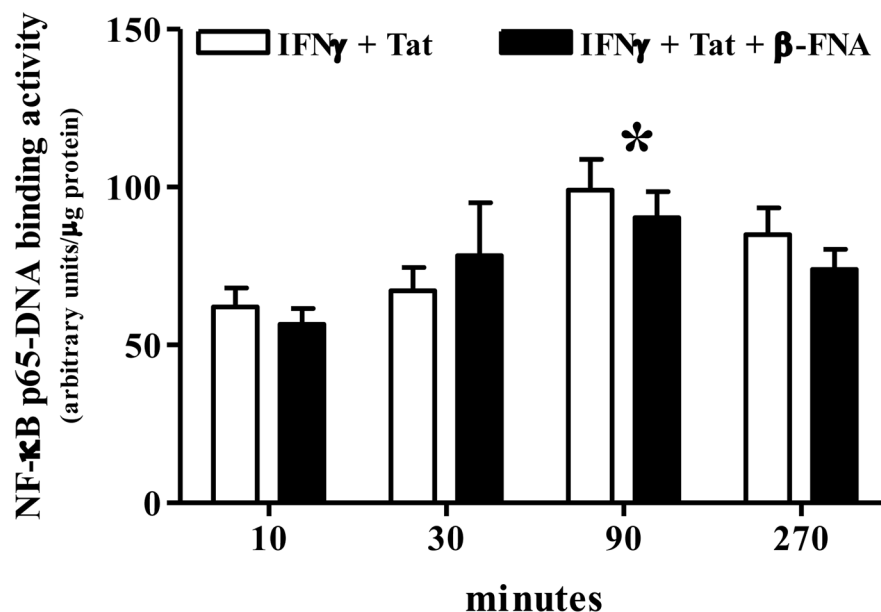




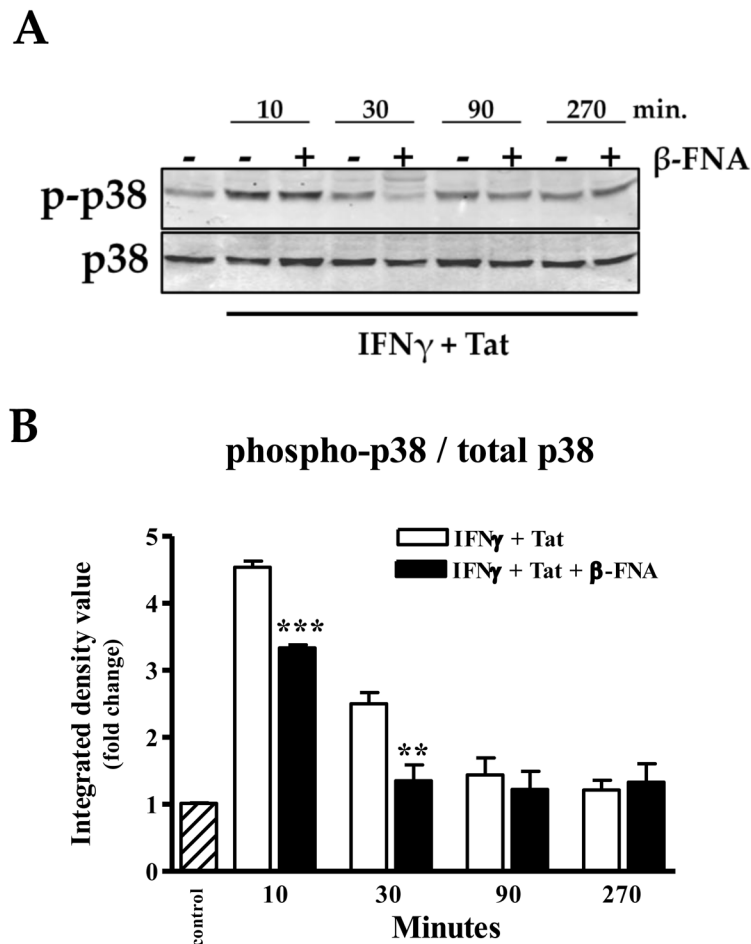
**Fig. 2.**  $\beta$ -FNA inhibition of IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>-induced CXCL10 protein expression in normal human astrocytes is not affected by co-exposure to either naltrexone (**Panel A**) or CTAP (**Panel B**). Normal human astrocytes were exposed to human recombinant IFN $\gamma$  (10 ng/ml) + HIV-1 Tat<sub>1-72</sub> (100 nM) alone or in the presence of  $\beta$ -FNA (10  $\mu$ M), naltrexone or CTAP (30 and 100  $\mu$ M), or  $\beta$ -FNA + naltrexone or CTAP for 24 h. CXCL10 protein levels in the media were then measured by ELISA. The data are expressed as % control (relative to IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>) and represent mean + S.E.M of 2–4 independent experiments; triplicate measures (wells) in each experiment. Data were analyzed using one-way ANOVA and Dunnett's post test comparison. \*  $p < 0.05$  vs. IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>



**Fig. 3.** IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>-induced CXCL10 protein expression in normal human astrocytes is NF- $\kappa$ B- dependent. CXCL10 expression was induced in normal human astrocytes by stimulating with human recombinant IFN $\gamma$  (10 ng/ml) + HIV-1 Tat<sub>1-72</sub> (100 nM) for 24 h. To assess the role of NF- $\kappa$ B, the inhibitor of NF- $\kappa$ B nuclear translocation, 50  $\mu$ M SN50 (or 50  $\mu$ M of the inactive control peptide, SN50 Mut), was added to cell cultures 1 h prior to stimulation. The data represent mean + S.E.M of 2 independent experiments; triplicate measures (wells) in each experiment. Data were analyzed using one-way ANOVA and Newman-Keuls post test comparison. \*  $p < 0.05$  vs. IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>



**Fig. 4.**  $\beta$ -FNA does not inhibit IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>, -induced NF- $\kappa$ B p65-DNA binding activity in normal human astrocytes. In the presence or absence of  $\beta$ -FNA (10  $\mu$ M), normal human astrocytes were exposed to human recombinant IFN $\gamma$  (10 ng/ml) + HIV-1 Tat<sub>1-72</sub> for 10–270 min. NF- $\kappa$ B activation was indicated by increased levels of the active (DNA-binding) form of NF- $\kappa$ B p65 in the nucleus. The data represent mean + S.E.M of 3 independent experiments. Two-way ANOVA ( $\beta$ -FNA treatment  $\times$  time) indicated a significant effect of time ( $p < 0.004$ ); whereas, there was neither a significant effect of  $\beta$ -FNA ( $p = 0.59$ ), nor an interaction ( $p = 0.62$ ). \*  $p < 0.05$  vs. 10 min as indicated by Bonferroni's multiple comparisons. Binding activity in unstimulated cells was  $43 \pm 7$  arbitrary units/ $\mu$ g protein.



**Fig. 5.**  $\beta$ -FNA inhibits IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub>-induced p38 activation in normal human astrocytes. In the presence or absence of  $\beta$ -FNA (10  $\mu$ M), normal human astrocytes were exposed to human recombinant IFN $\gamma$  (10 ng/ml) + HIV-1 Tat<sub>1-72</sub> for 10–270 min. Panel A): Western blot was used to determine levels of total p38 and phosphorylated-p38 (p-p38) in the cytosolic fraction. Panel B): Data for the densitometric analysis represent mean + S.E.M of 3–5 independent experiments. Integrated density values are presented as a ratio of p-p38/total p38 and expressed as fold change relative to unstimulated control. Data were analyzed using one-way ANOVA and Newman-Keuls post test comparison. \*\*  $p < 0.01$  vs. IFN  $\gamma$  + HIV-1 Tat<sub>1-72</sub> at 10 min; \*\*\*  $p < 0.001$  vs. IFN $\gamma$  + HIV-1 Tat<sub>1-72</sub> at 30 min.