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Morphine suppresses intracellular interferon- α expression in neuronal cells[★]

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Abstract

Interferon alpha (IFN- α) not only plays a key role in innate host immunity against infections but also is involved in the cellular functions of the central nervous system (CNS). In this study, we examined the impact of morphine on IFN- α expression in human neuronal cells (NT2-N). Similar to human immune cells, NT2-N cells also expressed IFN- α at both mRNA and protein levels. IFN- α expression in NT2-N cells, however, was inhibited by morphine. Naltrexone antagonized the inhibitory effect of morphine on IFN- α expression in NT2-N cells. The specific mu opioid receptor antagonist, Cys2, Tyr3, Arg5, Pen7-amide (CTAP), also blocked the morphine action on intracellular IFN- α expression. Investigation of the mechanisms involved in the morphine action showed that although morphine had little effect on the expression of key IFN regulatory factors (IRFs), morphine inhibited IFN- α promoter activation and suppressed the expression and phosphorylation of signal transducer and activator of transcription 1 (STAT1) in the neuronal cells. These findings provide direct *in vitro* evidence that opioids may impair neuronal cell-mediated innate protection in the CNS.

Keywords

Morphine; IFN- α ; NT2—N; Naltrexone; Interferon regular factor

1. Introduction

Opioids such as morphine exert a profound influence on immunomodulatory activity and affect regulatory actions of the central nervous system (CNS) (Carr and Serou, 1995; Hauser et al., 2007; Kapadia et al., 2005). Opioid abusers have been known to have higher incidence of infectious diseases, which may be directly related to impaired immune functions (Carpenter et al., 1994; McCarthy et al., 2001; Nair et al., 1997; Novick et al., 1989; Ochshorn et al., 1990; Peterson et al., 1993; Risdahl et al., 1998). The administration of morphine to rodents suppresses a variety of immune responses of major cell types in the immune system, including natural killer cells, T cells, B cells, macrophages and polymorphonuclear leukocytes (Bayer et al., 1990), and also inhibits antibody (Lockwood et al., 1994) and cytokine release (Hung et

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al., 1973; Wang et al., 2003). A significant suppression in interferon (IFN)- γ production by human blood lymphocytes was demonstrated in heroin addicts (Zaki et al., 2006). Acute morphine administration in rats suppresses splenic natural killer cell activity, mitogenic responses of blood lymphocyte, and *in vitro* production of the cytokines, such as interleukin-2, and IFN- γ (Kelschenbach et al., 2005; Peterson et al., 1989; Peterson et al., 1987; Wang et al., 2002). Moreover, it is now well established that opioids can modulate immune function (such as the inhibition of lymphocyte proliferation) via pharmacological activation of endogenous opioid receptors in the CNS (Bayer et al., 1990; Fecho et al., 1996; Hernandez et al., 1993).

In addition to affecting immune system, opioid abuse also impairs neurological function (Friedman and Eisenstein, 2004; Kapadia et al., 2005). While most of the studies have focused on the effect of opioids on adaptive immunity (Chang et al., 1998; Eisenstein and Hilburger, 1998; McCarthy et al., 2001; Roy et al., 1998), there is little information about whether opioids suppress neuronal cell-mediated innate immunity in the CNS. Innate immunity is the first line of the defense mechanism against invading pathogens. Interferons (IFNs) are critical elements involved in the innate host immunity against infections. IFNs are produced not only by the immune cells, but also by the CNS cells. Several recent studies have demonstrated that potential sources for IFN production in the CNS include neurons, astrocytes and microglia (Delhaye et al., 2006; Lafon et al., 2005; Prehaud et al., 2005). IFN- α expression was detected in primary neurons (Delhaye et al., 2006) as well as human neuronal cell line-NT2-N cells (Lafon et al., 2005). IFN receptors have been identified in the brain as well (Aguet, 1980; Aguet and Mogensen, 1983; Langer et al., 1996; Pestka et al., 1987). The existence of specific binding sites for IFNs within the CNS represents the link between IFNs and their effect on neuronal activity. Among IFN family members, IFN- α not only serves as an important element of the innate immunity in the CNS, but also participates in many important CNS functions (Dafny, 1998). Therefore, IFN- α may play a key role in the biological functions as well as the defense mechanism of the CNS.

Although it is known that the activation of opioid receptors by morphine impacts immune status in the CNS, there is little information about the direct interaction between opioids and intracellular innate host immunity in neuronal cells. We speculated that morphine, the active metabolite of heroin, compromises the neuronal cell-mediated innate immunity in the CNS. Thus, we investigated the impact of the activation of opiate receptor by morphine on endogenous IFN- α expression in human neuronal cells.

2. Materials and methods

2.1. Cell culture

Human neuronal cell line (NT2) was obtained from Ntera-2c1D/1 cells (ATCC CRL-1973). The cells were allowed to differentiate into neurons (Lafon et al., 2005). NT2 cells were maintained in Dulbecco's modified Eagles medium (DMEM) HG including 10% fetal bovine serum (FBS) with 100 units/ml penicillin plus 100 μ g/ml streptomycin (NT2 media) as previously described (Pleasure et al., 1992). For differentiation, NT2 cells (2×10^6) were seeded in a T75 cm² flask and treated with 10^{-5} M retinoic acid (RA) (Sigma-Aldrich, St. Louis, MO) in NT2 media at the concentration of 10^{-2} M in dimethyl sulfoxide (DMSO) twice a week for 5 weeks. Following RA treatment, the cells were replated (replate #1) into two T175 flasks in NT2 media. Cells were then (48 h later) washed twice with Hank's Balanced Salt Solution (HBSS) (Cellgro, Herndon, VA) and digested with 0.25% trypsin followed by mechanically dislodged, the floating cells were collected and washed with 5 ml of medium and replated on diluted Matrigel (1:30) in DMEM with 100 units/ml penicillin plus 100 μ g/ml streptomycin following the manufacturer's instructions. Cells were seeded at a density of 0.5×10^6 cells per well in a 24-well plate or 0.2×10^6 cells per 12 mm coverslip in NT2 media supplemented with 1 μ M cytosine arabinoside, 10 μ M fluorodeoxyuridine, and 10 μ M uridine (Sigma) (NT2-N

media). The fully differentiated NT2-N cells were used for the experiments at the 4th week during the culture with NT2-N media. The fully differentiated NT2-N cells express all well-defined neuronal markers including many neuronal cytoskeletal proteins, secretory markers, and surface markers (Pleasure et al., 1992). Analogous with primary neurons, NT2-N cells in cultures elaborate processes that differentiate into axons and dendrites. NT2-N cells express functional neuropeptides (Guillemin et al., 2000). We demonstrated that NT2-N cells express functional neuropeptide substance P and its receptor NK-1R (Li et al., 2003; Wang et al., 2004). Immature human NT2 cells grafted into mouse brain differentiated into neuronal and glial cells (Ferrari et al., 2000).

IM9 cell line (human B lymphoblasts) was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Peripheral blood samples were obtained from healthy subjects. The Institutional Review Board of the Children's Hospital of Philadelphia approved this investigation. Informed consent was obtained from the subjects. Peripheral blood mononuclear cells (PBMCs) and peripheral blood lymphocytes (PBL) were processed as described previously (Li et al., 2002). PBMCs were subjected to CD8⁺ T lymphocyte purification using anti-CD8 antibody-coated microbeads, according to the manufacturer's instruction (Miltenyi Biotec, Auburn, CA). Purified CD8⁺ T cells were cultivated in a 48-well plate precoated with the anti-CD3 antibody (3 µg/ml) in 10% FBS RPMI-1640 media containing interleukin-2 (50 U/ml) for 72 h. Monocytes were purified as described previously (Hassan et al., 1986). Monocyte-derived macrophages are monocytes that are maintained *in vitro* cultures for seven days. The human NK cell line (YTS) is a subclone of the YT lymphoid cell line derived from a patient with NK cell leukemia (Cohen et al., 1999). Human brain tissues used were obtained from National Neurological Research Specimen Bank (Los Angeles, CA).

2.2. Reagents and plasmids

Morphine sulfate (15 mg/ml) was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ). Naltrexone, mouse monoclonal antibody against microtubule-associated protein-2 (MAP-2) and rabbit polyclonal antibodies against actin, interferon regulatory factor 3 (IRF-3), IRF-7 and signal transducer and activator of transcription 1 (STAT1) were obtained from Sigma. Rabbit polyclonal antibody against IRF-5 was a gift from Dr. Rong-Tuan Lin (McGill University, Canada). CTAP (Cys2, Tyr3, Arg5, Pen7-Amide), a specific mu opioid receptor (MOR) antagonist, was obtained from Phoenix Pharmaceuticals, Inc. (Mountain View, CA). Mouse monoclonal antibody against IFN- α was purchased from Novus (Littleton, CO). Rabbit polyclonal antibody against IFN- α was purchased from Abcam Inc. (Cambridge, MA). Guinea pig anti-MOR antibody was obtained from Gramsch Laboratories (Schwabhausen, Germany). Mouse antibody to IFN- α/β receptor (IFNR) chain 2 was obtained from Chemicon International Inc. (Temecula, CA). TRITC-conjugated goat anti-mouse IgG antibody (Southern Biotechnology Associates Inc, Birmingham, AL) and Cy2-conjugated goat anti-guinea pig IgG antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit IgG antibodies were purchased from Jackson Immuno Research Laboratories Inc. (West Grove, PA). The plasmids (pIFN-A14-luc, wild-type IRF-7) and the control plasmid (pGL3) have been previously described (Lin et al., 2000).

2.3. Morphine treatment

Four weeks after incubation in 24-well plate, human NT2-N cells seeded at a density of 0.5×10^6 per well were treated with or without morphine at different concentrations (10^{-14} M to 10^{-8} M) for different time points (2–48 h). In the experiments designed to determine whether naltrexone or CTAP antagonizes the morphine action, we used naltrexone (10^{-8} M) or CTAP (10^{-8} M) to treat cells for 1 h followed by morphine treatment for additional 4 h. Cells were then collected for RNA and protein analysis.

2.4. RT-PCR and real time RT-PCR

Total cellular RNA was extracted from the cells (10^6) using Tri-Reagent (Molecular Research Center, Cincinnati, OH) as previously described (Guo et al., 2004). Total cellular RNA (1 μ g) treated with 1 μ l DNase I (Invitrogen, Carlsbad, CA) was subjected to reverse transcription using the reverse transcription system from Promega Corporation (Madison, WI). The real time RT-PCR for IFN- α mRNA was performed with the Brilliant SYBR Green QPCR Master Mix (Bio-Rad Laboratories, Hercules, CA). The specific oligonucleotide primers used were as follows: IFN- α : 5'-TTTCTCCTGCCTGAAGGACAG-3' (sense) and 5'-GTCATGATTCTGCTCTGACA-3' (anti-sense) (Shi X et al., 2003); GAPDH (the house keeping gene): 5'-GGTGGTCTCCTCTGACTTCAACA-3' (sense) and 5'-GTTGCTGTAGCCAAATTCGTTGT-3' (anti-sense). IFNR2.2: 5'-CCCCAAAGTCTTGAATTTTCATAACTT-3' (sense) and 5'-TGACAGTCAGTCCATGCATGGT-3' (anti-sense) (Gazziola et al., 2005). MOR: 5'-ACCAACATCTACATTTTCAACCTT-3' (sense) and 5'-CAGTACCAGGTTGGATGAGAG-3' (anti-sense) (Wan et al., 2006). The oligonucleotide primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). After PCR amplification, the samples were separated by 2% ultra pure agarose gel electrophoresis (Gibco-Grand Island, NY).

2.5. Transfection and luciferase assays

Transfection of plasmid containing IFN promoter (pIFN-A 14-luc) and wild-type IRF-7 were carried out with FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) as recommended by the manufacturer. Luciferase activities in cell lysates were quantified using a Luciferase Assay System (Promega) and a TD-20/20 Luminometer (Turner Designs, Inc., Sunnyvale, CA). The results were presented as relative light units (RLU) normalized by the total proteins in the cell lysates. For IFN- α promoter-luciferase reporter experiments, the transfection was performed in 24-well plates with 0.5×10^6 cells in NT2 media. The cells were transfected with the plasmid pIFN-A 14-luc alone or combined with IRF-7. FuGENE 6 was used at ratio of FuGENE 6: the plasmid 6:1 (μ l: μ g) in a total volume of media of 50 μ l NT2 media. Cell culture without morphine treatment was used as the control. Morphine (10^{-12} M to 10^{-8} M) was added to culture 2 h pretransfection. Cells were then lysed in 1 $\times$ report lysis buffer (Sigma) for luciferase assay 24 h post-transfection.

2.6. Immunofluorescence assay

The NT2-N cells were cultured on glass coverslips at a density of 0.2×10^6 /well in 24-well plates. The NT2-N cells were washed with $1 \times$ cold PBS (with Ca^{2+} and Mg^{2+}) twice. Cells were fixed at 4 $^{\circ}\text{C}$ in 4% paraformaldehyde–4% sucrose in PBS for 20 min and then permeated in cold methanol (100%) for additional 10 min followed by 0.2% Triton X-100 for additional 10 min. Cells were blocked in Block Solution (Pierce, Rockford, IL) for 1 h at room temperature. In order to examine co-expression of IFNR and MOR, mouse monoclonal antibody to IFNR (1:100) and guinea pig polyclonal antibody against MOR (1:100) (Gramsch Laboratories, Schwabhausen, Germany) were used as the primary antibody. After washing 5 times with $1 \times$ PBS, the cells were incubated with secondary antibodies: TRITC-conjugated goat anti-mouse (red) (1:250) and Cy2-conjugated goat anti-guinea pig (green) (1:100) antibodies for 1 h. After washing 5 times with $1 \times$ PBS, the cells were mounted on glass coverslips in mounting media (Biomed, Foster City, CA) and viewed with a fluorescence microscopy (Zeiss, Germany). Hoechst 33342 was used for nuclei morphology. For staining of MAP-2 and IFN- α , cells were incubated with the primary antibodies: mouse anti-MAP-2 and IFN- α monoclonal antibodies (1:100) for 1 h at room temperature and TRITC-conjugated goat anti-mouse antibody was used as the secondary antibody. Mouse IgG1 and guinea pig IgG (1:100) were isotype controls for the primary antibodies described above.

2.7. Western blot analysis

Total cell lysates from the NT2-N cells treated with or without morphine (10^{-10} M) were prepared using the lysis buffer consisted of 10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM sodium-orthovanadate, 20 μ M 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.2% Nonidet P-40, 5 μ g/ml leupeptin, phosphatase inhibitor cocktail and protease inhibitor cocktail (Sigma). Protein concentrations were determined by DC protein assay kit (Bio-Rad). Proteins were resuspended in NuPAGE LDS Sample Buffer (NOVEX), heated for 5 min at 95 °C and then equivalent amounts of protein (50 μ g) for each sample were separated in a 10% Bis—Tris Gel in a NuPAGE Running Buffer with 0.25% Running Buffer Antioxidant for 50 min at 200 V. Proteins were transferred to the nitrocellulose membranes (Bio-Rad) in NuPAGE Transfer Buffer at 100 V for 1 h. The membranes were blocked by 5% fat-free milk in PBST (0.05% Tween 20 in PBS) for 1 h and incubated with appropriate optimal diluted antibodies for β -actin (1:3,000) for 1 h at room temperature or with rabbit polyclonal antibodies to IFN- α (1:250), IRF-3 (1:500), IRF-5 (1:500), IRF-7 (1:500), and STAT1 (1:500) overnight at 4 °C in the blocking solution. After washing 3 times for 15 min each with PBST, the membrane was incubated with appropriate HRP-conjugated goat anti-rabbit (1:10,000) IgG antibodies in blocking solution for 1 h at room temperature and washed 3 times as described above. The bound antibodies were developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) according to the manufacturer's instruction and exposed to basic Autorad film. Pre-stained molecular markers (Bio-Rad) were used to determine molecular weight of immunoreactive bands. For densitometry analysis, films were scanned using Quantity-One program (Bio-Rad Laboratories, Hercules, CA) and the fixed cursor area centered over each band was assessed for pixel density with normalization to actin signal.

2.8. Nuclear extraction and STAT 1 ELISA assay

NT2-N cells seeded at a density of 0.5×10^6 per well in a 24-well plate were treated with or without morphine at 10^{-10} M for 30 min. Cells (2×10^6) were washed with $1 \times$ cold PBS twice and lysated with nuclear extraction buffer A (Nuclear Extraction Kit, Panomics, Fremont, CA). Culture vessels were mixed well following centrifugation at 14,000 g for 3 min at 4 °C. The supernatant consisting of cytoplasmic extract was discarded. The remaining nuclear extract was suspended in the nuclear extract buffer B (Nuclear Extraction Kit, Panomics, Fremont, CA). The extract was mixed well and then centrifuged at 14,000 g for 5 min at 4 °C. The supernatant of nuclear extract was aliquoted and stored at -80 °C until analyzed by STAT1 ELISA Assay Kit following the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA).

2.9. Statistical analysis

Where appropriate, data were expressed as mean $\pm$ SD. For comparison of the mean of the two groups (morphine and/or naltrexone or CTAP treated versus untreated control cells), statistical significance was assessed by ANOVA with the appropriate post hoc test—Bonferroni/Dunnett's test and statistical significance was considered as $P < 0.05$.

3. Results

3.1. IFN- α expression in NT2-N cells

In the first set of experiments, we examined and compared IFN- α expression in both human immune and CNS cells. As shown in Fig. 1, all the immune cells such as IM9 (B lymphocyte cell line), CD8⁺ T cells, PBL, macrophage and the natural killer cell line (YTS) expressed IFN- α mRNA. Similar to the immune cells, NT2-N cells expressed IFN- α mRNA at the levels that are equal or higher than the immune cells (Fig. 1). IFN- α mRNA was also detected in human brain tissues (Cingulate cortex and cerebellum) (Fig. 1). In addition, we examined IFN- α

expression during the course of NT2-N cell differentiation. There was a gradual increase in IFN- α mRNA expression during the course of NT2 differentiation (Fig. 2A). Fully differentiated NT2-N cells (9 weeks in cultures) also expressed intracellular IFN- α proteins as demonstrated by immunofluorescence (IF) assay (Fig. 2B). The neuronal characteristics of NT2-N cells were evidenced by the expression of the neuronal marker, MAP-2 (Fig. 2B).

3.2. NT2-N cells express both MOR and IFNR

Since biological activities of morphine and IFN- α are mediated through their receptors, we examined whether NT2-N cells express both MOR and IFN receptor (IFNR). Fully differentiated NT2-N cells expressed both MOR and IFNR mRNA as determined by RT-PCR (Fig. 3A). Dual labeling IF assay using the specific antibodies revealed that there was co-expression of both MOR and IFNR in NT2-N cells at protein levels (Fig. 3B). The specificity of the guinea pig anti-MOR antibody was demonstrated by others (Bigliardi-Qi et al., 2000, 2004; Bigliardi et al., 2003) and confirmed in our experiments using AV12 cells transfected with the human MOR gene containing plasmid (Zhang et al., 1998) and untransfected AV12 cells (data not shown).

3.3. Morphine suppresses IFN- α expression in NT2-N cells

We next examined whether morphine affects IFN- α expression in NT2-N cells. Morphine suppressed IFN- α mRNA expression in NT2-N cells (Fig. 4A). The highest inhibition by morphine was observed at the concentration of 10^{-10} M (Fig. 4B). In addition, the greatest down-regulation of IFN- α mRNA was observed at 4 h post-treatment with morphine at 10^{-10} M (Fig. 4B). We further examined whether IFN- α protein expression is also inhibited in morphine-treated NT2-N cells. Morphine-treated cells (24 h) had lower level of IFN- α protein than untreated NT2-N cells (Fig. 4C). The morphine action on IFN- α , however, was antagonized by naltrexone, a prototypical opioid-receptor antagonist (Fig. 5), while naltrexone alone had little effect on IFN- α expression (Fig. 5). In order to determine whether MOR is the primary receptor involved in the morphine action, we used a specific MOR antagonist (CTAP) to block the morphine action on IFN- α expression. Similar to naltrexone, CTAP completely abolished morphine's effect on intraneuronal IFN- α expression, while CTAP alone had little effect on IFN- α expression (Fig. 5).

3.4. Morphine inhibits IFN- α promoter activity and STAT protein expression

In order to investigate the mechanisms involved in morphine-mediated IFN- α down-regulation, we examined whether morphine inhibits IFN- α promoter activity in the NT2-N cells. Morphine, when added to NT2-N cells transfected with IFN- α promoter (pIFN-A 14-luc), inhibited the activation of IFN- α promoter as indicated by decreased IFN- α promoter-driven luciferase expression (Fig. 6A). The pattern of the inhibition of IFN- α promoter by morphine at different concentrations is similar to that observed in Fig. 4, showing that morphine suppressed IFN- α expression. We also examined whether IRF-induced IFN- α promoter activation is affected by morphine. IFN- α promoter (pIFN-A 14) was efficiently activated by the introduction of IRF-7 gene into NT2-N cells (Fig. 6B). However, IRF-7-induced IFN- α promoter activity was significantly suppressed by the morphine treatment (Fig. 6B). Since STAT1 and IRF family members (IRF-3, 5 and 7) play key roles in signaling of IFN- α activation, we investigated the role of morphine in the regulation of these factors. NT2-N cells treated with morphine had lower levels of STAT1 protein expression than the control cells (Fig. 6C). In addition, morphine inhibited the phosphorylation of STAT 1 (Fig. 6D). Morphine, however, had little effects on IRF-3, IRF-5 and IRF-7 protein expression in NT2-N cells (data not shown).

4. Discussion

Although opiates, especially morphine and heroin, have been widely studied with regards to their effects on the immune system as well as the neurological system (Friedman and Eisenstein, 2004), little is known about the direct effect of morphine on the intracellular innate immunity of neuronal cells. We, thus, examined the impact of morphine on the expression of IFN- α , a key element of innate defense mechanisms. To address whether morphine has negative effects on endogenous IFN- α expression in neurons, we first examined whether human neuronal cells express IFN- α at both mRNA and protein levels. Our data is in agreement with the recent reports (Delhay et al., 2006; Lafon et al., 2005; Prehaud et al., 2005) that human neuronal cells express IFN- α . Interestingly, the levels of IFN- α expressed by NT2-N cells are comparable to those by human immune cells, suggesting that IFN- α may have a key role in neuronal functions and protection against viral infections. We next demonstrated that NT2-N cells express MOR and IFNR at both mRNA and protein levels. The co-expression of both MOR and IFNR in the neuronal cells provides a biological basis for reciprocal interactions between opioids and IFNs in the CNS. We hypothesized that opioids suppress intracellular IFN- α expression in the neuronal cells. This hypothesis is based on the evidence that there exists a well-recognized relationship between addictive drugs such as heroin and increased incidence of deficient innate immune response. For instance, morphine has the ability to reduce the levels of circulating endogenous IFN- α (Geber et al., 1975; Hung et al., 1973). Opioids also inhibited the expression of anti-viral cytokines, such as IFN- α and IFN- γ in the immune system (Eisenstein et al., 1993; Homan et al., 2002; Kelschenbach et al., 2005; Peterson et al., 1987). Morphine suppresses Sendai virus-induced IFN- α production by PBMCs (Nair et al., 1997). Morphine also inhibits the release of IFN- α/β by Sindbis virus-infected monocytes (Stoll-Keller et al., 1997), and negatively regulates IFN- γ promoter activity in activated murine T cells (Wang et al., 2003). Our observation that morphine has the ability to suppress IFN- α expression in human neuronal cells, provides an additional data to support the notion that opioids have the ability to impair host cell-mediated innate immunity.

Biological activities of morphine are mediated through its receptor. It has been documented that in neuronal cells, morphine binds to a G-protein-coupled MOR to induce analgesia (Law and Loh, 1999; Loh and Smith, 1990). Our observation that human neuronal cells express MOR is in agreement with the studies by others (Bani-Yaghoub et al., 2001; Horner and Zadina, 2004) as well as our earlier studies (Wan et al., 2006; Wang et al., 2004). The expression of MOR on NT2-N cells is also supported by the evidence that naltrexone and the MOR specific antagonist, CTAP, completely antagonized the morphine action on IFN- α expression. Although CTAP appeared to facilitate IFN- α expression, this impact, however, is not statistically significant. In the present study, we observed a U shaped dose—response curve of NT2-N cells following morphine treatment. This finding might be due to MOR desensitization caused by the high concentration of morphine. Several studies have shown that the opioid agonists such as morphine have the ability to induce internalization and down-regulation of MOR expression in human neuroblastoma cells (Baumhaker et al., 1993; Chakrabarti et al., 1997; Horner and Zadina, 2004). At the cellular level, loss of responsiveness to an agonist is a recurrent theme in the field of G-protein coupled receptor including MOR (Horner and Zadina, 2004).

The major pathway of intracellular signaling used by IFN- α/β and their receptors is by activating the signal transducer and activator of transcription (STAT). In addition, the interferon regular factor (IRF) family has an important role in the innate host response to viral infections (Barnes et al., 2002). Three IRFs (IRF-3, 5 and 7) are direct transducers of virus-mediated signaling of type I IFN pathway and play a critical role in the activation of type I IFN genes (Au and Pitha, 2001; Barnes et al., 2001; Marie et al., 1998). Particularly, IRF-3 and 7 execute important functions in rapid induction of IFN- α A 14 promoter expression, a central

event in establishing the innate anti-viral responses (Barton and Medzhitov, 2003; Honda et al., 2005; Sen, 2001). While both IRF-3 and IRF-7 are the primary players in the activation of IFN- β and IFN- α , respectively, IRF-5 was also characterized as an important factor participating in the induction of IFN- α/β (Barnes et al., 2001). In contrast to our earlier study showing that morphine withdrawal inhibited IRF-5 and IRF-7 expression in human hepatocytes (Li et al., 2007; Wang et al., 2005), we observed that morphine had little effect on the expression of these IRF family members in the neuronal cells (data not shown). This discrepancy could be due to the difference in the cell types used in our earlier work and the present study. Although morphine had no direct effect on the expression of IRFs, the inhibition of STAT1 expression and phosphorylation in the neuronal cells by morphine indicated the involvement of STAT pathway in the action of morphine on IFN- α expression. Furthermore, our finding that morphine has the negative effect on the IFN- α promoter (IFN- α A14) activation with or without IRF-7 stimulation provides an additional molecular mechanism for the morphine action on the expression of IFN- α . Thus, it is clear that morphine-mediated alteration of IFN signaling pathway contributes to intracellular IFN- α deficiency, which may compromise neuronal cell functions and protection.

Because IFN- α is involved in many important CNS functions and has been implicated as a key neuromodulator, the down-regulation of IFN- α expression in the neuronal cells by morphine may constitute a critical mechanism for the CNS dysfunction often seen among opioid abusers. Our data that morphine suppressed intracellular IFN- α expression in neuronal cells not only provide direct evidence to support the involvement of IFN- α in opioid-mediated neuronal functions, but also indicate that opioid may impair the innate immune protection for neuronal cells. Therefore, defining factors that modulate IFN- α expression and type I IFN pathways in the CNS is of importance, which may lead to the development of new therapeutic strategies for treating opioid-mediated immune suppression and/or neuronal dysfunction in the CNS. Nevertheless, future studies are needed to determine the impact of morphine on IFN- α expression in the *in vivo* microenvironment where multiple cell types and cellular factors interact in the CNS.

Abbreviations

CTAP, Cys2, Tyr3, Arg5, Pen7-amide
 IFNs, Interferons
 IFN- α , Interferon alpha
 IFN- β , Interferon beta
 IFN- γ , Interferon gamma
 IRF-3, interferon regular factor 3
 IRF-5, interferon regular factor 5
 IRF-7, interferon regular factor 7
 IRFs, interferon regular factors
 MAP-2, microtubule-associated protein-2
 MOR, mu opioid receptor
 PBMCs, Peripheral blood mononuclear cells
 STAT1, signal transducer and activator of transcription 1.

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Fig. 1.

IFN- α expression in both the immune and CNS cells. RNA was extracted from the human immune cells and CNS cells as indicated. Total cellular RNA (1 μ g) of each cell types was subjected to real time RT-PCR using the specific primer pairs for IFN- α (371 bp) and GAPDH (127 bp). The amplified products were then subjected to electrophoresis on 2% ultra pure agarose gel. One representative result of 3 independent experiments is shown.

Fig. 2.

IFN- α expression in NT2-N cells. (A) IFN- α expression during the course of differentiation of NT2 to NT2-N cells *in vitro*. Undifferentiated NT2 cells (day 0 in culture) were cultured in RA media up to 9 weeks, and RNA was collected every 2 or 3 weeks followed by the real time RT-PCR analysis. (B) Immunofluorescence analysis of IFN- α protein expression in differentiated NT2-N cells. NT2-N cells phase was captured (a) and stained with mouse monoclonal antibody to MAP-2 (b, Red) and mouse IgG1 was used as an isotype control (c). Mouse monoclonal antibody to IFN- α was used to detect IFN- α in NT2-N cells (d, Red). The specimens were examined by a fluorescence microscope with magnification of $\times 200$. One

representative result of 3 independent experiments is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

IFNR and MOR expression in NT2-N cells. Total cellular RNA extracted from NT2-N cells was analyzed by RT-PCR to detect IFNR (205 bp) and MOR (441 bp) mRNA (A). Sizes are estimated from DNA ladder (100 bp fragments) co-electrophoresed as the marker. NC: Negative control (the same NT2-N without reverse transcription). In addition, NT2-N cells were plated on coverslips and stained with antibodies to IFNR (Red) and MOR (Green) (B). Hoechst 33342 (Blue) was used as the nuclear marker. Upper panel: the antibody-stained NT2-N cells; Lower panel: control antibody-stained NT2-N cells. G.P: Guinea pig. The specimens were examined by a fluorescence microscope with magnification of $\times 200$. One representative result of 3 independent experiments is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Effect of morphine on IFN- α expression in NT2-N cells. NT2-N cells were treated with morphine at different concentrations as indicated (A) and the effect of morphine (10^{-10} M) on IFN- α expression was analyzed at different time points post-treatment (B). Total cellular RNA was then extracted and subjected to real time RT-PCR. The data shown are the mean $\pm$ SD of triplicate cultures, representative of 3 independent experiments (*, $P < 0.05$, morphine vs. control). In addition, equal amount (50 μ g) of total proteins extracted from NT2-N cells treated with morphine at indicated time points were subjected to Western blot assay (C). IFN- α (19 kD) density in each sample was normalized by β -actin (42 kD). One representative result of 3 independent experiments is shown.

Fig. 5.

Effect of the opioid antagonists on the morphine action on expression of IFN- α in NT2-N cells. Naltrexone (10^{-8} M) and CTAP (10^{-8} M) were added to NT2-N cell cultures for 1 h prior to morphine treatment. Total cellular RNA was then extracted 4 h post-treatment and subjected to the real time RT-PCR. The data shown are the mean $\pm$ SD of triplicate cultures, representative of 3 independent experiments (*, $P < 0.05$, morphine vs. control).

Fig. 6. Effect of morphine on IFN- α promoter activation and STAT1 expression. (A, B) Effect of morphine on IFN- α promoter activation. NT2-N cells were treated with morphine at indicated concentrations for 2 h before the transfection with the plasmid containing IFN- α promoter (pIFN-A14-luc) (A) or IFN- α promoter plus IRF-7 plasmid (B). Morphine remained in the cultures during and after the transfection. Luciferase activities in the cell extracts were determined 24 h post-transfection. The data shown are the mean $\pm$ SD of triplicate cultures, representative of 3 independent experiments (*, $P < 0.05$, morphine vs. control). (C) Effect of morphine on the expression of STAT1. Equal amounts (50 μ g) of total proteins extracted from the NT2-N were subjected to Western blot assay using antibodies specific to STAT1, and Actin. Arrows indicate the positions of STAT1 (91 kD). One representative result of 3 independent experiments is shown. (D) Effect of morphine on the phosphorylation of STAT1. Total nuclear protein was extracted from NT2-N cells treated with or without morphine (10^{-10} M) for 30 min. The levels of phosphorylated STAT1 levels were determined by ELISA assay of intranuclear STAT1 protein. The data shown are the mean $\pm$ SD of triplicate cultures, representative of 3 independent experiments (*, $P < 0.05$, morphine vs. control).